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VOL. 10 æ 1958 æ NO. 4

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THE SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION

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The Scandinavian Journal of Clinical and Laboratory Investigation publishes original articles closely associated with clinical research and based on laboratory investigations. Pure methodological work of importance for clinical research will also be accepted for publication. Authors in the Scandinavian countries should send their manuscripts to one of the editors in their respective countries. Their addresses are printed above.

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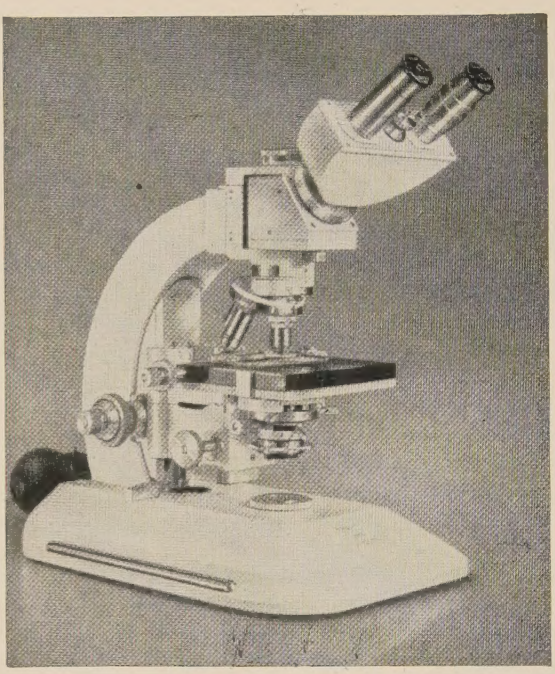

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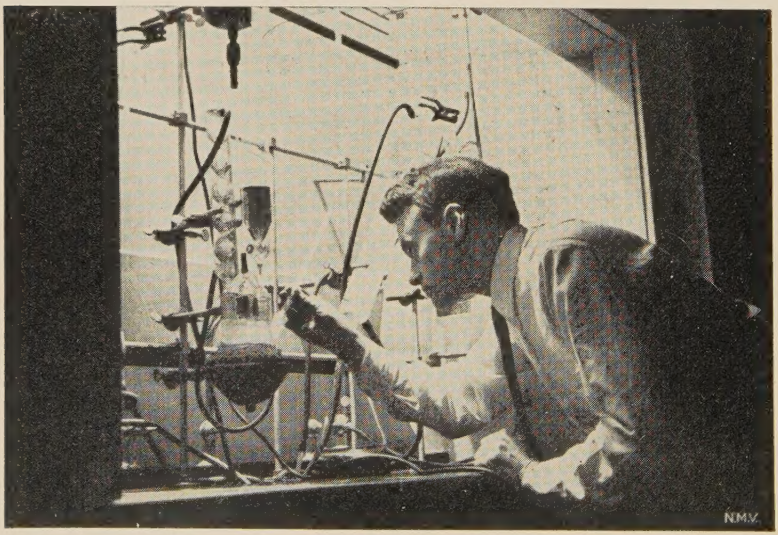

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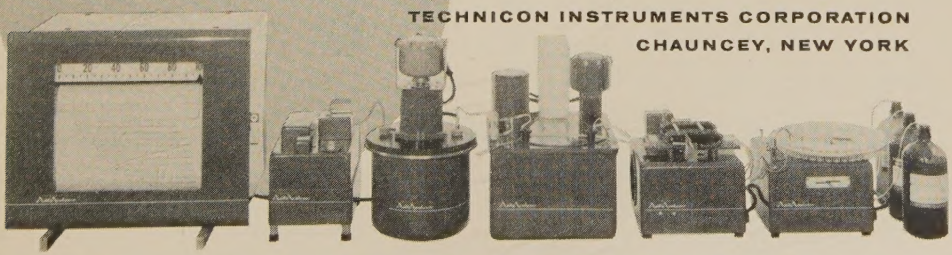

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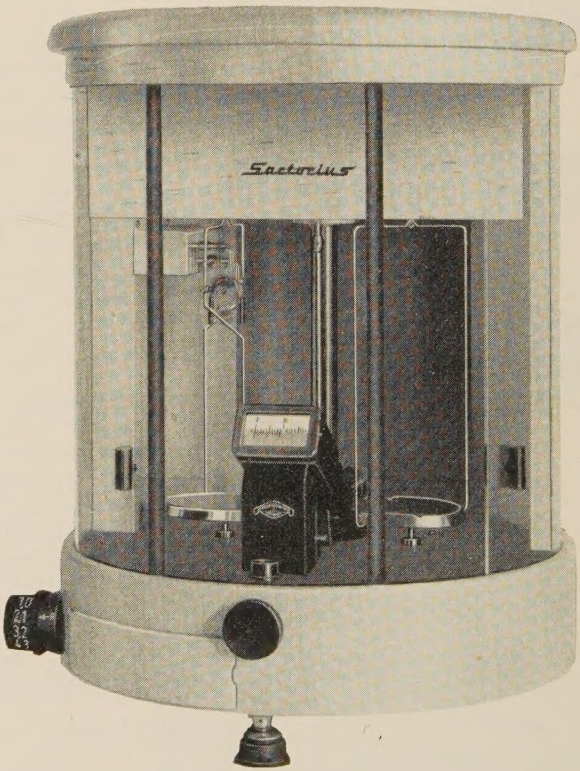

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Vol. 10 · 1958 · No. 4

ndinav. J. Clin. & Lab. Investigation 349—354, 10, 1958.

ACID SERUM-PHOSPHATASE LEVELS FOLLOWING MASSAGE OF THE PROSTATE

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(Received for publication June 7, 1958)

Earlier reports by Hock & Tessier (1949) by Daniel & van Zyl (1952) presented idence of augmented activity of acid serum-osphatases in some patients following assage or palpation of the prostate. These vestigators had only one determination of zyme activity before massage and were us unable to estimate spontaneous fluctua-ons in the phosphatase concentration. The esent work includes such measurements d furthermore has a control group.

MATERIALS AND METHODS

Preliminary experiments on eleven male patients l to the following procedure employed on twenty-ven male patients eighteen of whom received assage of the prostate whereas nine served as ntrols:

A thick ordinary needle was placed in a cubital in and physiological saline containing 1500 units heparin per liter was infused at a rate of about e milliliter per minute for the first two hours. Blood samples were obtained after disconnection needle and infusion tube. The first two milliliters blood were discarded before each sample was taken, and sampling was done 10, 5 and 1 minute before the "massage period". During this latter period, lasting for 2 minutes, eighteen patients (test group) were subjected to a firm massage per rectum of all accessible parts of the prostate; in the remaining nine patients (control group) a finger was inserted in the rectum but the prostate was not palpated. In the first nine test-patients samples were then taken at 3, 10, 20, 30 and/or 60 minutes and in six of these nine cases also at 120 and/or 240 minutes after massage. In the nine last test-patients and in all controls the periods were 2, 5, 10, 20, 60 and 240 minutes after massage.

Care was taken to separate erythrocytes from serum by repeated centrifugation and three sera with macroscopic haemolysis were discarded. Determinations of acid serum-phosphatase (ASP) levels were made according to King (1951). Two tests and two serum controls on each sample as well as daily phenol standard were assayed and the color read against water in a Beckman B spectrophotometer at 765 mμ, using 10 mm cells.

The concentration of enzymes is given in *King-Armstrong units per liter serum* — hereinafter abbreviated to KA. 99 % of normal males have values between 8 and 46 KA. By multiplying with 0.177 the King-Armstrong unit per liter serum may be converted into the unit *μmol substrate hydro-*

lysed per minute per liter serum $\left(\frac{\mu mol}{min \cdot l}\right)$.

The analytical standard deviation on a duplicate was 2.0 KA and the error of the mean 1.4 KA (about 7 %).

RESULTS

Test Group

The eighteen patients were divided into the following three groups, according to usual clinical criteria, and mainly according to the palpatory findings. *Group N* comprised three patients (aged 26 to 31 years) with normal prostates, *group H* twelve patients (aged 57 to 81 years) with benign hypertrophia (diffuse, firm, elastic enlargement of the gland without lateral infiltration, a normal ASP, microscopy of the surgically removed prostate showing typical benign hypertrophia in five cases), and *group C* three patients (aged 62 to 78 years) with cancer of the prostate (palpation in all cases showed very hard nodules in the gland and in two cases lateral infiltration; in one case osteoblastic metastases were found in the costae, and in another the diagnosis was confirmed at autopsy.)

In the period before massage sixteen patients had ASP values in the range 11—33, one had 9—15 and one (in group C) 46—47.

One case (in group H) showed an increase from 11 to 24 KA, otherwise the greatest rise was from 9 to 15 KA and the greatest fall from 21 to 14 KA.

The first and the last value obtained before massage were compared according to Fisher (1938) and as no systematic fluctuations could be demonstrated it was considered justifiable to compare the values in each patient after massage with the individual means of the values before massage.

The means of the *values following massage* were calculated partly from the values obtained during the first ten minutes after massage — which time equals the premassage period — and partly from the first sixty minutes.

With the method of Fisher previously used the individual *means* in each group in the period before massage were compared with corresponding means in the first 10 minutes after massage and in the first 60 minutes. No significant differences were found. Thus no *systematic* effect of massage can be detected. Each patient may, however, be examined *per se* and the means after massage compared with the mean value before.

First the individual variances (s^2) before and after massage (for either 10 or 60 minutes period) were compared by the F-test (v^2 test). If no significant difference was found in each case, the difference of the means was tested in the usual way.

If the variances differed significantly the value of $t = \frac{\bar{x}_1 - x_2}{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$ was calculated. The corresponding degrees of freedom are less than $n_1 + n_2 - 2$, but no formula has been reported for the exact calculation when population samples are very small and the significance of each t value can only be roughly estimated. The results are shown in Table 1.

Control Group

The nine patients in this group were divided into two groups according to the palpatory findings. *Group N:* three patients (25—45 years of age) with normal prostates *group H:* six patients (64—80 years of age)

massage of prostate. Heavy type indicate significant deviations, i. e. $P \leqq 0.05$. Where the variances of the individual means are statistically different the degrees of freedom are uncertain (less than the value calculated as $f = n_1 + n_2 - 2$) and consequently the probability is only roughly estimated.

Condition of prostate gland	Patient no.	Abbreviated mean value (and number of determinations)			Comparison between means							
					before vs. first 10 min.				before vs. first 60 min.			
		before massage	first 10 min. after massage	first 60 min. after massage	Δ	| t |	f	P	Δ	| t |	f	P
Normal	1	24_2	20_2	19_4	**—4**	$s_1^2 = s_2^2$	=0	significant	**—5**	6.351	<4	probably significant (0.02—0.05 for f=2)
	2	32_3	25_2	20_5	—7	10.511	<3	possibly significant (0.002–0.01 for f=2)	**—12**	4.043	6	0.002—0.01
	3	26_3	27_3	27_4	+1	4.010	<4	possibly significant (0.02—0.05 for f=3)	**+1**	2.654	5	0.02—0.05
Hypertrophy	4	29_3	28_2	28_2	—1	0.778	3	0.40—0.60	—1	0.773	3	0.40—0.60
	5	11_3	18_2	19_4	+7	2.347	3	~0.10	**+8**	3.902	5	0.01—0.02
	6	16_3	14_2	14_3	—2	0.932	3	0.40—0.60	—2	1.133	4	0.20—0.40
	7	25_3	28_2	27_4	+3	1.773	3	0.10—0.20	+2	1.781	5	0.10—0.20
	8	23_3	21_2	22_4	—2	0.418	3	0.60—0.80	—1	0.124	5	>0.80
	9	25_3	24_2	22_4	—1	1.526	3	0.20—0.40	—3	2.470	5	0.05—0.10
	10	17_3	18_3	18_5	+1	1.190	4	0.20—0.40	+1	0.283	6	0.60—0.80
	11	27_2	29_3	29_3	+2	0.905	3	0.40—0.60	+2	0.905	3	0.40—0.60
	12	12_3	20_3	20_5	**+8**	3.704	4	0.02—0.05	**+8**	4.618	6	0.002—0.01
	13	21_3	18_2	20_4	—3	10.039	<3	possibly significant (0.002—0.01 for f=2)	—1	0.683	5	0.40—0.60
	14	18_3	18_3	21_5	0	—	—	—	+3	1.372	<6	(0.20—0.40 for f=5)
	15	12_3	25_3	27_5	**+13**	11.754	4	<0.001	**+15**	4.297	6	0.002—0.01
Cancer	16	47_3	54_2	53_4	+7	11.980	3	0.001—0.002	**+6**	16.163	5	<0.001
	17	27_3	34_3	33_5	+7	2.585	4	0.05—0.10	**+6**	3.125	6	~0.02
	18	13_3	14_3	15_5	+1	0.759	4	0.40—0.60	+2	1.545	6	0.10—0.20

Table II. *Control group. Comparison of each patient's mean ASP value before insertion of finger in rectum (no massage) with means of values obtained in the first 10 and first 60 minutes following insertion.*

Condition of prostate gland	Patient no.	Abbreviated mean value (and number of determinations)			Comparison between means							
					before vs. first 10 min.				before vs. first 60 min.			
		before insertion	first 10 min. after insertion	first 60 min. after insertion	Δ	|t|	f	P	Δ	|t|	f	P
Normal	19	25_3	26_2	24_4	+1	0.347	3	0.60—0.80	—1	0.398	5	0.60—0.80
	20	26_3	28_3	26_5	+2	1.043	4	0.20—0.40	0	—	—	—
	21	30_3	33_2	33_4	+3	0.869	3	0.40—0.60	+3	0.777	5	0.40—0.60
Hypertrophy	22	28_3	29_2	28_3	+1	0.218	3	>0.80	0	—	—	—
	23	37_3	35_3	32_5	—2	0.828	4	0.40—0.60	—5	1.784	6	0.10—0.20
	24	37_3	39_3	37_5	+2	0.551	4	0.60—0.80	0	—	—	—
	25	24_3	24_3	28_5	0	—	—	—	+4	0.828	6	0.40—0.60
	26	32_3	38_3	43_5	+6	2.449	4	0.05—0.10	+11	2.239	6	0.05—0.10
	27	28_2	25_2	26_4	—3	0.235	2	>0.80	—2	0.304	4	0.60—0.80

h hypertrophy of the benign type. The ial ASP was found to be normal in all e cases.

n the period *before insertion of finger in rum* no systematic fluctuations could be nd between first and last value. One case group H showed an increase from 19 to KA, otherwise the greatest rise was 20 27 and the greatest fall 42 to 37 KA.

For each group the individual means of first ten and first sixty minutes *following ertion of the finger* were calculated and pared to the individual means before in- tion. As before, no systematic deviations ld be demonstrated.

Finally the intra-individual comparison is wn in Table 2. The greatest rise from 32 43 KA (no. 26), was mainly caused by unexplained high value of 56 KA, which s the only abnormal ASP value in the ntrol group.

DISCUSSION

Hock & Tessier (1949) stated that the d serum-phosphatase level following mas- ge of the prostate increased in 17 out of 20 ients, but the authors give no information ncerning the analytical technique and error their Bodansky-procedure. A closer exa- nation of their data reveals that some de- tions were very small and in a few cases decrease is seen following massage. Ten ients, however, showed a seemingly signi- ant rise in phosphatase value and in two es with pronounced benign hypertrophy augmentation was very marked: 0.2 → 4.9 1 0.0 → 15.5 Bodansky units.

In seven normal persons Daniel & van (1952) found no rise in acid serum- sphatase following diagnostic palpation ot massage) of the prostate. Samples were obtained before and one hour as well as 24 hours after palpation. Neither did 3 cancer patients and 21 patients with hypertrophy show any significant rise. In a group of three cases of hypertrophy, however, a significant mean rise was observed: 39 before, 97 one hour after and 54 King-Armstrong units per liter serum 24 hours after palpation.

In the present investigation three normal patients and twelve with hypertrophy of the prostate showed no *systematic* increase nor decrease when the individual mean acid serum-phosphatase level following energetic massage of the prostate was compared to the pre-massage mean. Three proved or probable cancer patients gave consistently higher mean values following massage.

Taking each patient *per se* (see Table 1) a comparison of the ASP mean following massage with the pre-massage mean, demonstrated a significant *decrease* in two of three patients with a normal prostate and possibly in one with hypertrophy. One normal subject showed a very small *rise* and three with hypertrophy a significant increase as was also the case with two out of three cancer patients (highest single values 54 and 39 KA respectively). One cancer patient had abnormally high ASP values before massage as well as after, but in no other case was the upper normal limit (46 KA) exceeded. Three patients with normal prostates and six with hypertrophy served as controls and none showed significant deviations.

These results are in contrast with the results of Daniel & van Zyl (1950): They could demonstrate no fluctuations in normal persons and cancer patients whereas we found an unexplained decrease in the first and an increase in the second group following massage. The increase in some cases of hyper-

trophy is in agreement with the two earlier reports, even if the augmentations are rather small in our patients.

The method employed here does not permit a profitable comparison of *single* values after massage with the mean value before: Three patients in the control group showed a significant increase at some time following the insertion of a finger in the rectum.

Previous and present results do not indicate that a special trend in ASP values following massage of the prostate is of value in the differential diagnosis of diseases of of the prostate.

In some cases a considerable augmentation is undoubtedly seen and consequently blood for determination of acid serum-phosphatases should not be sampled for the first 24 hours following massage or palpation of the prostate.

It is possible that determination of tartrate sensitive acid phosphatases would yield more valuable results, as this fraction of the total phosphatase activity probably is a relevant expression of concentration of prostatic enzyme alone.

We do not feel fully convinced that the massage is a harmless procedure. Our last patient in the test group, 67 years of age, had a very large prostate with benign hypertrophy and some urinary retention, but no history of acute complete retention. Immediately after massage he spontaneously expressed a feeling of great relief. Eight hours later, however, the patient developed complete urinary retention and it was necessary to catheterize with a metal catheter and regularly thereafter with ordinary rubber catheters. The patient underwent a complicated electroresection nine days after the massage and then declined steadily and died sixteen days after the operation. Whether the massage was the cause of the urinary retention is impossible to say.

CONCLUSION AND SUMMARY

In three patients with a normal prostate, twelve patients with hypertrophy and three with cancer of the prostate

1) no *systematic* fluctuations were found in the mean acid serum-phosphatase level following energetic massage of the prostate.

2) Examination of the data of each patient revealed a decrease in two normals and an increase in one normal, three subjects with hypertrophy and in two with cancer of the prostate.

3) The procedure as employed does not seem of diagnostic value.

4) Blood determination of acid serum-phosphatase should not be sampled during the 24 hours immediately following massage or palpation of the prostate.

ACKNOWLEDGEMENT

The authors' thanks are due to chief physician Torben Geill, M. D. and professors Svend Hansen, M. D. and H. C. Gram, M. D., for encouragement during the work.

Dr. Gerda Seidelin, M. D., is sincerely thanked for much help with the statistical principles.

Finally it is the authors' pleasure to acknowledge the help of technicians Mrs. Birgit Hovøre, Miss Kirsten Pedersen and Miss Else Mortensen.

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METHOD FOR THE ESTIMATION OF PHENYLPYRUVIC ACID IN URINE WITH SOME EXAMPLES OF ITS USE IN DIETARY TREATMENT OF PHENYLPYRUVIC OLIGOPHRENIA

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(Received for publication April 25, 1958)

While qualitative demonstration of phenyluvic acid in the urine is simple (Følling, 4), there have been various difficulties in rying out quantitative estimations.

The green color that appears after adding iron salt ought to be suitable for coloriry, but a simultaneous precipitate of sphates interferes very much with readS. This precipitate is dissolved by adding d, but too much acid will decrease the en color. Further, the picture is complied by the varying color of the urine itself d the color of other organic acids which react with the iron.

Several attempts have been made to find etter method for quantitative estimation phenylpyruvic acid in urine. Penrose & astel (1937) used 2,4 dinitrophenylhydrae and estimated the red color that apred after addition of NaOH. It has been wn that this method gives too high values ause of other ketone bodies in the urine erry & Woolf, 1952). Further, the blank not be carried out and seems to be large variable. Berry & Woolf have worked a method based on the extraction of urine h ether. This method does not seem to be l either. The many processes necessary ore the reaction can be carried out often to loss of material and variable results. We have therefore tried to adapt the original method so that it could be read in a photometer.

We do not know exactly what occurs in the reaction between iron and phenylpyruvic acid. Apparently a bond occurs between the two substances which is immediately broken as more phenylpyruvic acid is transformed. It is assumed that the enol form reacts with iron and the reaction thus takes place on the acid side of the neutral point. If the urine is alkaline the keto form predominates. The urine must therefore be acid. It has been shown that if the urine is made alkaline the readings decrease at once and it takes about an hour to reattain maximal values if the urine is then made acid again. The optimal pH is about 2,2. If the pH comes to 1 the reaction becomes weaker. If the urine contains much phosphate, even at pH 2,2 it may be difficult to prevent precipitation of phosphates. Precipitation must therefore be carried out first. This was found to be difficult. The easiest method was to precipitate the phosphates with MgO in substance and filter. The urine was then acidified with HCl and stood for an hour before the reaction was carried out. Precipitation of phosphates was necessary only when large volumes of urine had to be used for each analysis.

METHOD

Possible variations in the pH of the urine are eliminated first by adding conc. HCl to pH about 2.2 (Merck Universal Indicator paper). A suitable volume of urine is taken (0.1—2.0 ml) and 0.1 M glycine-HCl buffer pH 2.2 is added to 2.25 ml. Stand for 1 hour. Then 0.25 ml of an aqueous solution of $Fe(NH_4)_2SO_4 \cdot 12\,H_2O$, 50/100 w/v is added. Maximal extinction is read in an Uvispec spectrophotometer at wave length 600 mmy, slit 5, 1 cm cell agaist glycine-HCl buffer.

In order to eliminate the persisting yellow color which is not due to phenylpyruvic acid, the cell is left standing for one hour. The minimal extinction is then read and deducted from the first reading. Standard curve, Fig. 1.

The reaction between iron and phenylpyruvic acid occurs rapidly. Maximal color development occurs in about 1 minute, remains at the maximal level for 10—15 seconds and then fades first rapidly and later more slowly in 3/4 to 1 hour. When the green color is gone, a yellow color of varying intensity remains unchanged for a long time. If the temperature is above 20° C the reaction occurs so rapidly that the maximum can hardly be read. This can be avoided by first storing the sample in a refrigerator. Checks agreed very well both for standard solution and urine.

The reaction is very sensitive, 0.1—0.2 ml urine is usually used. With decreasing amounts of phenylpyruvic acid the volume of urine must be increased, up to 2.0 ml. With this volume of urine down to 1—2 mg can be demonstrated in a 24 hr. sample. Phosphoric acid must then usually be precipitated first with MgO.

CLINICAL OBSERVATIONS

Several attempts have been made to treat patients with phenylpyruvic oligophrenia with a phenylalanine-poor diet, thus reducing the phenylalanine content of the blood (see references).

Such a diet must include enough protein to maintain the nitrogen balance and the phenylalanine must be reduced to the minimum necessary for maintenance. As most

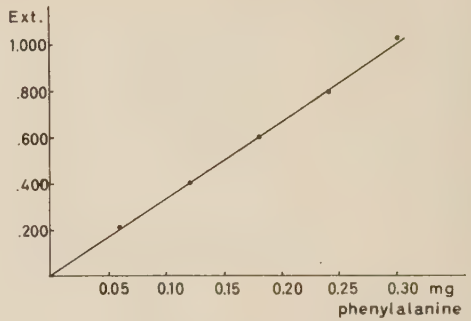

Fig. 1. Standard curve. Phenylalanine in absolute values.

proteins contain 4—6 per cent phenylalanine, a hydrolysate from which it has been removed, usually a casein hydrolysate, may be used. But during hydrolysis and purification not only phenylalanine but also some tyrosine, cystine and tryptophan are removed. These amino acids must therefore be added afterwards. Another but very expensive method is to use a suitable mixture of pure amino acids.

With this treatment the phenylalanine content of the blood decreased considerably and the excretion of pyruvic acid in the urine stopped even while the phenylalanine content of the blood was still relatively high, showing that examination of urine alone in these patients is not sufficient to control the effect of the treatment.

Several of the clinical symptoms improved. The skin became more normal, the hair darker, the epileptic seizures ceased in many cases, and the electroencephalographic changes became less noticeable. The mental condition on the other hand did not improve so much although some cases became more alert and more normal behavior was noticed. This sometimes persisted for some time after the discontinuation of the treatment.

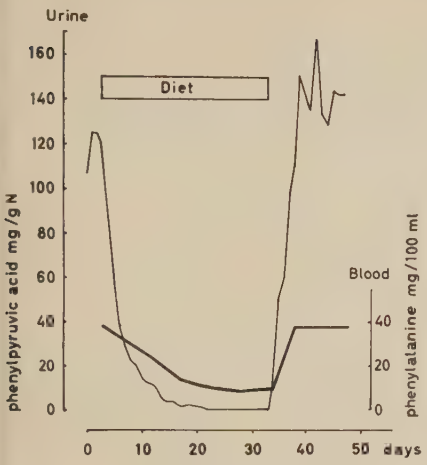

Fig. 2. Patient No. 1.
—— : Excretion of phenylalanine in 24 hr. urine calculated as mg/g nitrogen excreted.
——: Phenylalanine in blood in mg/100 ml.

In cooperation with the Pediatric Clinic have treated two patients with phenylruvic oligophrenia with a phenylalanine-or diet. The excretion of phenylpyruvic d in the urine was determined by the de-ibed method and the phenylalanine content the blood by a method developed by dling & Løken.

Patient 1 was a girl of 11 years, who had been y retarded since birth. The diagnosis was made the Pediatric Clinic when she was 10 months old. At the start of dietary treatment she was com-tely dull without contact with her surround-s. She had a characteristic smell.

The phenylalanine content of the blood was ng/100 ml, and she excreted large quantities of enylpyruvic acid in the urine. EEG showed nges in the deep medial structures.

She was put on a diet consisting of: casein drolysate 35 g, l-tyrosine 2.5 g, l-cysteine 1.0 g, ryptophan 0.25 g, milk 200 g. Wheat starch, atoes, sugar, margarine, vegetables and a little it were added, so that she got 1560 calories in In addition she had minerals and vitamins.

In 14 days the phenylalanine content of her blood creased from 38 to 14 mg/100 ml, and the excretion phenylpyruvic acid in the urine decreased in 3

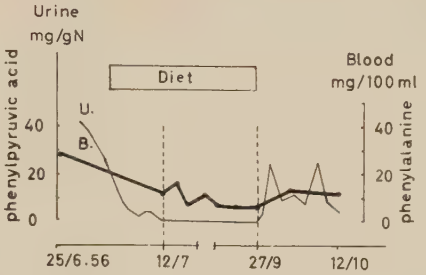

Fig. 3. Patient No. 2.
—— : Excretion of phenylalanine in 24 hr. urine calculated as mg/g nitrogen excreted.
——: Phenylalanine in blood in mg/100 ml.

weeks to 0. The phenylalanine in the blood decreased further to 9 mg/100 ml.

After dietary treatment for 1 month she was again given ordinary food, and in 4 days the phenylalanine in the blood rose to 38 mg/100 ml. The phenylpyruvic acid in the urine was then even higher than before treatment began. The only clinical improvement noted was that her characteristic smell was less pronounced than before. This lack of clinical improvement would perhaps be expected in a patient so markedly affected and in whom the condition had lasted so many years. The period of treatment was also very short.

Patient no. 2 was a 15-month-old girl. She was somewhat mentally retarded, but there were no other very marked symptoms. EEG was normal. The phenylalanine content of the blood was 29 mg/100 ml and she excreted some phenylpyruvic acid in the urine.

She was put on a diet consisting of: casein hydrolysate 25 g, l-tyrosine 1.8 g, l-cystine 0.5 g, dl-tryptophan 0.35 g, milk 200 g. Wheat starch, sugar, margarine, potatoes, vegetables and oranges were added, so that she got a total of 990 calories. In addition, like the first patient, she was given minerals and vitamins. The first week she brought up some of the food, but subsequently she kept it down.

In just over a week the excretion of phenylpyruvic acid in the urine decreased to 0 and the phenylalanine in the blood fell to 12 mg/100 ml. During the rest of the period of treatment, phenylpyruvic acid could not be demonstrated in the urine. The blood values were rather variable but finally decreased to just below 8 mg/100 ml.

After 3 months the diet had to be stopped because no more casein hydrolysate was available. After a few days of normal food, phenylpyruvic acid was found in the urine, but not so much as previously. The phenylalanine content of the blood did not return to the original high level. Both her parents and the ward staff noticed definite improvement in her intelligence while in hospital.

A blood test taken 3 months after discharge showed that the phenylalanine content of the blood had increased to 24 mg/100 ml. The mental improvement lasted only for some weeks after discharge, subsequently there was progressive deterioration.

DISCUSSION

Reviewing the recent literature, it must be said that the dietary treatment of this condition is still in the experimental stage. Restriction of phenylalanine in the diet of these patients can reduce the concentration of phenylalanine in the blood considerably and prevent the excretion of abnormal metabolites in the urine. Improvement in the clinical condition, especially the mental development, is less noticeable. This indicates that it is not only the action of abnormal metabolites on a healthy brain that produces symptoms, changes have probably taken place which are very slowly reversible, or irreversible.

Treatment should therefore begin as early as possible, preferably immediately after birth and must probably be continued for a very long time, at least throughout the period of development, probably all the patient's life. In the milder cases it might be sufficient merely to reduce the intake of foods containing phenylalanine.

The practice of dietary treatment in its present form would involve many difficulties. Casein hydrolysate has an unpleasant smell and taste and it is likely that enthusiasm for the diet will decrease with increasing intelligence. It would not be possible to get a more or less normal older child to take such a diet for a long time unless it was denied access to other food. Even if the diet tasted good there would be difficulties as the possibilities for variation are small. It must therefore be said that dietary treatment in its present form shows little promise as an effective form of treatment of these patients.

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ADDENDUM

After the conclusion of this investigation a method based on the same principle is published by The, Fleury & Vink (Clin. Chim. Acta, *2,* 424, 1957).

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DETERMINATION OF CONJUGATED 17-HYDROXYCORTICOSTEROIDS IN HUMAN PERIPHERAL BLOOD

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(Received for publication April 30, 1958)

Hydrolysis of conjugated corticosteroids their metabolites with acids or specific ymes is today a standard procedure when e hormones are assayed in urine. In plas- however, the determination of conju- d 17-hydroxycorticosteroids (17-OHCS) not so far been widely practised. Quanti- ve methods, based on the color reaction Silber & Porter's (1954) sulfuric acid ethanol — phenylhydrazine reagent have n shown to be an accurate and simple ns of assaying free 17-OHCS in plasma. atively few attempts have been made to mate the conjugated plasma steroid frac- with the same reaction.

Klein, Papadatos, Fortunato & Byers 55) used hydrolysis with 25—37 per cent rochloric acid in the determination of jugated plasma 17-OHCS. Bongiovanni 54), Bongiovanni & Eberlein (1954) and giovanni, Eberlein, Grumbach, VanWyk Clayton (1955) performed the hydrolysis liver β-glucuronidase. In their earlier eriments Bongiovanni *et al.* eliminated rfering chromogens from the hydrolysis ture by dialysing against 12 volumes of per cent methanol in water. In later lies the procedure was simplified by omit- dialysis. Instead the plasma proteins e precipitated by 95 per cent ethanol and inated by filtration or centrifugation. The clear residue was evaporated and redissolved in water. After the extraction of the free 17-OHCS with dichloromethane the hydrolysis of the conjugates was performed. Subsequently, after completed hydrolysis the 17-OHCS were extracted by dichloromethane. The final determination of both free and conjugated 17-OHCS was carried out according to Nelson & Samuels method (1952), in which the plasma extracts are chromatographed on a florosil column prior to the color reaction. This purification procedure was developed by Bongiovanni *et al.* in order to eliminate the nonspecific chromogens formed during the hydrolysis.

It has been reported by Louis, Eiler, Streeten & Conn (1956) that the addition of glucose to the urine of normal individuals before enzymatic hydrolysis with β-glucuronidase resulted in a great elevation of the Silber-Porter reacting material. By adding sodium bisulfite to the urine-enzyme-glucose mixture prior to incubation, the interfering material was selectively eliminated.

In our studies on plasma 17-OHCS we have found that sodium bisulfite can also eliminate the interfering substances formed in the plasma during β-glucuronidase hydrolysis. In this paper we report our simplified procedure for determining conjugated plasma 17-OHCS.

TECHNIQUE

0.5 ml of 1 M potassium dihydro-phosphate, pH 4.5, 400 units of bacterial β-glucuronidase [1] in one ml of redistilled water and 0.3 ml of 5 per cent sodium bisulfite (15 mg) were added to 5 ml of heparinized plasma. The final pH of the mixture was 6.0—6.2. The incubation was performed in glass-stoppered extraction tubes at 37° C in darkness for 48 hours. After this period the total plasma 17-OHCS were extracted with 25 ml of purified and redistilled dichloromethane. After this the procedure was the same as the free 17-OHCS method of Peterson, Karrer & Guerra (1957). The free plasma 17-OHCS were determined simultaneously from another 5 ml plasma portion. The difference between the total and free plasma 17-OHCS was the conjugated 17-OHCS content in the plasma investigated.

Both series, free and total 17-OHCS, were performed with their own standards and blanks, which were treated quite in the same way as the plasma samples. An aqueous solution of cortisol containing 1 μg per 5 ml was used as standard. This solution was not stored longer than two weeks at room temperature. The blanks were made from 5 ml of redistilled water. The β-glucuronidase solution was freshly prepared every week and stored in a refrigerator. The 5 per cent sodium bisulfite solution was also prepared weekly and stored in a similar way.

RESULTS AND DISCUSSION

Effect of sodium bisulfite

Our first experiments to determine plasma 17-OHCS using β-glucuronidase hydrolysis confirmed the findings of Bongiovanni *et al.* We found that the appearance of interfering material made an exact estimation of the 17-OHCS content with the Silber-Porter reagent impossible. When 15 mg of sodium bisulfite were added to 5 ml of plasma prior to hydrolysis, the non-specific chromogens were, however, eliminated and a typical spectral absorption curve of cortisol obtained. In all instances the curve showed a maximum at 400 to 410 mμ and the interfering chromogens, which at 350—370 mμ already give a strong absorption, were absent.

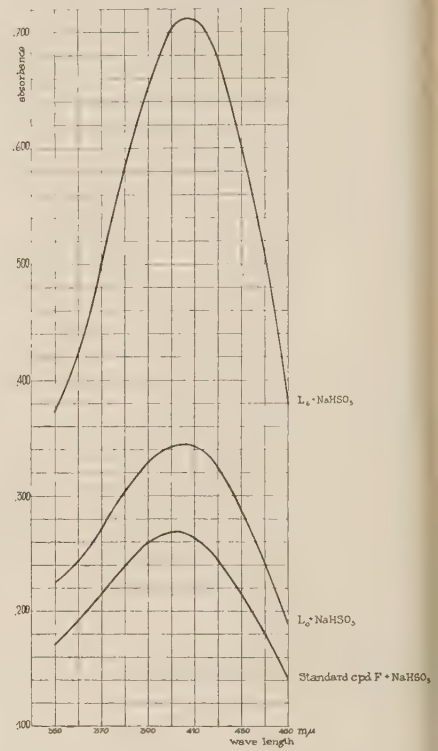
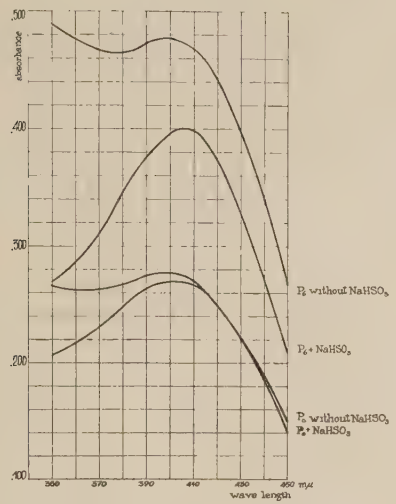


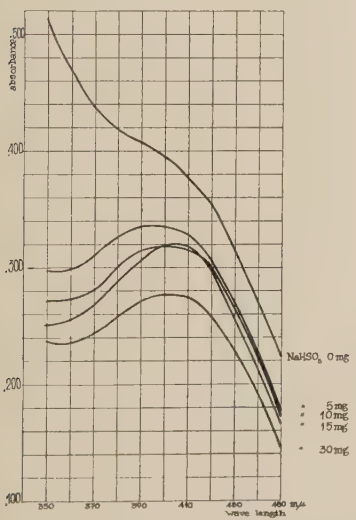
Fig. 1. Spectral absorption curves of phenylhydrazine-reacting material in two plasmas hydrolyzed with β-glucuronidase in the presence of sodium bisulfate and a cpd. F standard treated in the same way.
L_0 = Plasma L before ACTH administration.
L_6 = Plasma L after 6 hours intravenous ACTH infusion.

Fig. 1 shows the typical spectral absorption curves of two plasma samples and a cortisol reference standard after β-glucuronidas

[1] In this study only bacterial β-glucuronidase (Sigma) with an approximate activity of 25,000—30,000 units per g was used. The dry β-glucuronidase was stored in a vacuum exsiccator above silica gel at a low temperature.



2. Spectral absorption curves of phenylhydra-reacting material in two plasmas hydrolyzed β-glucuronidase without or with addition of sodium bisulfite.
= Pooled plasma collected before ACTH administration.
= Pooled plasma collected after 6 hours' intravenous ACTH administration.



3. Effect of sodium bisulfite concentration on spectral absorption curve of phenylhydrazine-eacting material in plasma hydrolyzed with β-glucuronidase.

hydrolysis in the presence of sodium bisulfite. One of the plasma samples (L_0) was drawn before an intravenous ACTH capacity test, the other (L_6) after six hours intravenous ACTH infusion.

Fig. 2 presents an analogous series of the absorption curves of two hydrolyzed plasma samples both with and without sodium bisulfite. These curves demonstrate clearly the selective effect of sodium bisulfite in the elimination of interfering chromogenic material formed during the β-glucuronidase hydrolysis.

The effect of varying concentrations of sodium bisulfite on the non-specific chromogens was also studied (Fig. 3). The addition of 15 mg sodium bisulfite to 5 ml plasma seemed to give optimal results but also smaller amounts, *i. e.* 10—12.5 mg to 5 ml produced about the same results.

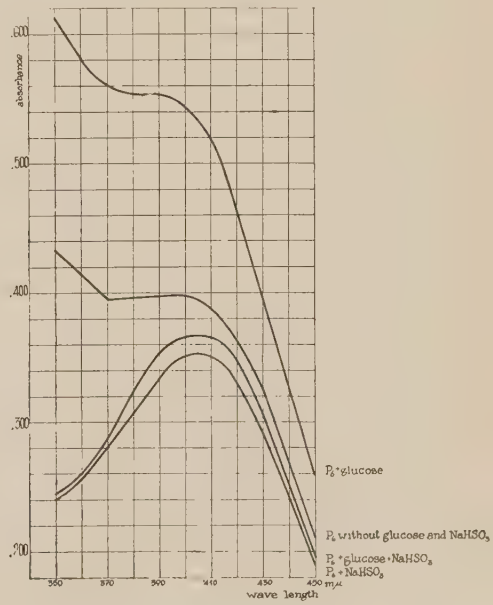


Fig. 4. Effect of glucose (193.5 mg/100 ml) and sodium bisulfite on the spectral absorption curve of phenylhydrazine-reacting material in a plasma hydrolyzed with β-glucuronidase.

Table I. *Effect of glucose and sodium bisulfite on the yield of total plasma 17-OHCS in 48 hours' hydrolysis with bacterial β-glucuronidase.*

Sample	Total plasma 17-OHCS μg/100 ml	
	Pooled normal plasma	Pooled ACTH plasma
Plasma without glucose and sodium bisulfite	22	58
Plasma + sodium bisulfite ..	21	45
Plasma + glucose (193.5 mg/100 ml)	50	90
Plasma + glucose (193.5 mg/100 ml) + sodium bisulfite	22	47

Table II. *Amount of bacterial β-glucuronidase required for maximal yield of total plasma 17-OHCS.*

β-Glucuronidase Units/5 ml plasma	Total plasma 17-OHCS μg/100 ml	
	Pooled normal plasma	Pooled ACTH plasma
200	18.3	53.0
300	19.6	54.0
400	19.6	55.8
600	18.4	55.8
800	22.0	51.3
1000	17.8	46.5
1500	23.2	50.4

Effect of glucose

Louis *et al.* (1956) claimed that the presence of glucose in the urine to be hydrolyzed resulted in a great elevation of the apparent 17-OHCS values. In order to establish the possible effect of glucose on the total plasma 17-OHCS values we added this sugar in excesss to pooled plasma (193.5 mg/100 ml, dissolved in saturated sodium benzoate solution) and performed the enzyme hydrolysis in parallel samples without and with sodium bisulfite. The results of this series are demonstrated in Fig. 4 and Table I. It can be seen that the addition of glucose results in falsely high total 17-OHCS values. This false increase of the 17-OHCS values does not, however, appear if sodium bisulfite is added prior to hydrolysis.

In this connection it may be mentioned that sodium bisulfite is also able to eliminate the interfering effects of solvent impurities (dichloromethane). This shows that the effect of sodium bisulfite is not specifically directed against glucose and its degradation products.

Enzyme concentration

The amount of β-glucuronidase needed for a maximal yield of total plasma 17-OHCS was also studied (Table II). An increase of the enzyme amount above 400 units per 5 ml plasma did not clearly increase the yield of total 17-OHCS. As lower β-glucuronidase concentrations also gave fairly good results, there were no indications for increasing the enzyme amount above 400 units per 5 ml of plasma.

Several authors (*e. g.* Bongiovanni & Eberlein, 1955) have shown that a hydrolysis time of 48 hours is sufficient for the liberation of conjugated plasma 17-OHCS. Experiments concerning this point will not be discussed in this study. Equally, it is a well established fact that the pH optimum of bacterial β-glucuronidase is about pH 6.0—6.2 (*e. g.* Buehler, Katzman, Doisy & Doisy, 1949). In our experiments we have constantly used a pH of 6.0—6.2 in the plasma hydrolysis mixture. This was achieved by the addition of potassium dihydro-phosphate buffer to the hydrolysis mixture.

he technique for the determination of ugated plasma 17-OHCS described in paper has been used in some of our cal studies (Krusius & Oka, 1958; Kru-, Oka & Kärkelä, 1958; Oka, 1958; Oka Krusius, 1958).

SUMMARY

he addition of sodium bisulfite to a buf-d plasma — β-glucuronidase mixture r to incubation has made it possible to rmine the conjugated plasma 17-OHCS simple method without laborious puri-ion procedures. The following observa-s have been made:

The addition of sodium bisulfite to the rolysis mixture eliminates selectively the -specific chromogens interfering in the er-Porter reaction.

A specific spectral absorption curve of ma cortisol with a maximum at 400— mμ is obtained.

The spectrophotometer readings at 410 are generally lower, never higher, than out the addition of sodium bisulfite.

The results are valid also in cases ted with ACTH.

Sodium bisulfite eliminates the inter-ng effect of glucose added in excess to plasma prior to hydrolysis.

The background absorption caused by ent impurities can also be eliminated by um bisulfite.

400 units of bacterial β-glucuronidase e been found to be sufficient for the rolysis of 5 ml plasma.

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TUBULAR REABSORPTION OF GLUCOSE DURING PREGNANCY

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(Received for publication May 8, 1958)

Robertson & Gray (1953) have determined the renal tubular reabsorption of glucose in a pregnant woman with true renal diabetes. Their findings indicate fundamental changes affecting the mechanism of reabsorption of glucose with a great increase in reabsorptive capacity in early pregnancy and a decrease towards term. Donato & Turchetti (1955) have published a material indicating the opposite.

The present paper is an attempt to provide further information on renal tubular treatment of glucose by simultaneous investigation of renal threshold and maximal tubular reabsorption (TmG). The results indicate that the renal threshold for glucose is diminished during pregnancy, whereas TmG is not. The low threshold is probably due to an increase in glomerular filtration rate.

MATERIALS AND METHODS

The material, cf. Table I, consists of 13 adult, female persons, 10 of them patients from the Department of Obstetrics and Gynecology, Aarhus University, the Surgical Department K, Aarhus Municipal Hospital, and the Medical Department of Marselisborg Hospital, Aarhus. Three of the persons are healthy women taken into hospital just for the examination. All had a normal, unrestricted diet during the days preceding the experiment. The hospitalized persons were kept in bed with permission to visit lavatory and to leave the bed to wash. The pregnant group consists of (a) one woman in the 1st month of pregnancy with a renal diabetes of at least 10 years' duration, in hospital just cured for a minor bacterial skin infection, (b) 5 normal women in the 3rd—4th month of pregnancy, all of them hospitalized, but without signs of disease during the days preceding the experiment, and (c) two normal women in the 7th and 9th month of pregnancy.

Collection and analysis of samples

To ensure a satisfactory hydration of the person investigated, 0.5 to 1 liter of fluid is given during the evening before the experiment. In the morning, 30 minutes before the beginning of the experiment, cf. Table II, 0.5 to 1 liter of water is given to the fasting person. At zero hour the bladder is emptied through an indwelling catheter (portion A). Shortly thereafter a blod specimen (No. 1) is drawn from a superficial vein of the forearm, and a permanent inulin infusion is instituted. The infusion solution consists of sterile water containing 2 g inulin and 0.72 g sodium chloride per 100 ml. Subsequent blood samples are withdrawn from the other forearm which is kept warm by covering with towels. 30 minutes after the beginning of the inulin infusion the bladder is emptied again and washed twice with 50 ml sterile water. The combined urine and wash water from this portion (B) is discarded. The exact time is taken. Blood specimens are withdrawn between the bladder emptyings and time is taken. After 3 clearance periods, each one of about 10 minutes' duration, infusion of a sterile solution of 20 per cent glucose is added to the inulin infusion and given through the same cannula. While

Table I. *Data on the persons examined.*

son o.	Journal No.	Name	Age years	Height cm.	Weight kg.	Remarks
			Non-pregnant			
	D 375/57	R. S.	27	151	48.0	Obs. pro salpingo-oophoritis chron.
	E 583/57	A. P.	31	160	49.5	Prolapsus disci intervertebralis sequelae.
	E 698/57	B. J.	24	156	58.0	Insufficientia isthmica uteri obs.
		L. C.	30	164	52.0	Not hospitalized.
	D 1306/57	P. D.	29	164	54.0	Insufficientia isthmica uteri.
			Pregnant			
	A 1571/57	L. J.	19	168	64.7	Diabetes renalis. Graviditas m. 1.
	G 354/57	J. F.	23	158	66.2	Graviditas m. 3, obs. pro abortus imm., no bleeding.
	H 264/57	J. C.	23	170	60.0	Graviditas m. 4, obs. pro abortus imm., no bleeding.
	K 9319/57	E. P.	27	157	49.8	Graviditas m. 4, depressio mentis.
	K 1606/57	E. J.	26	160	64.0	Graviditas m. 3, depressio mentis.
	K 10637/58	K. S.	27	160	61.0	Graviditas m. 3, depressio mentis.
		I. S.	37	173	71.0	Not hospitalized. Graviditas m. 9.
		A. J.	15	160	63.5	Not hospitalized. Graviditas m. 7.

inulin infusion is given constantly at a rate of
nl per minute throughout the whole experiment,
glucose infusion rate is elevated stepwise from
nl per minute through 6 to 8 ml per minute.
om each clearance period urine and wash water
combined. During the first half of the experi-
nt the person is given about 500 ml of water to
nk in addition to the above mentioned quantities
order to keep the diuresis as constant as pos-
e. A typical experimental record is shown in
ble II.

Inulin analyses for clearance determination are
formed according to the method of Goldring,
asis, Ranges & Smith (1940). As blank values
plasma and urine the inulin-free samples 1 and
respectively, are used, (cf. Table II). All ana-
s of one experiment are carried out on one day,
with the same yeast suspension and the same
ch of diphenylamine reagent. Calculation of inu-
concentration is based on a fixed correlation be-
en light extinction and inulin concentration,
= 1.000 corresponding to 94 mg inulin per 100 ml
od plasma or urine dilution.

Glomerular filtration rate (GFR) is calculated as the mean of inulin clearances for 3 clearance periods before glucose loading. An exception is made for the two persons in late pregnancy, in whom GFR is determined as an average of all inulin clearances.

The analyses of glucose in blood plasma and urine are carried out according to the method described by Somogyi (1945 a, b), without any correction for the insignificant reduction originating from inulin. Throughout the present investigation 1 ml 0.005 N sodium thiosulphate is found to be equivalent to 0.135 ± 0.003 mg glucose in the portion titrated.

The plasma concentrations used in the calculation of clearances and tubular loads are found by interpolation between the plasma concentrations measured, the time used being 2 minutes prior to the middle of each clearance period as a correction for "delay time".

The maximal tubular reabsorption of glucose (TmG) is calculated as a mean of all observations at plasma glucose levels where glucose reabsorption

Table II. *A typical experimental record (L. C.).*

Time	Blood sample No.	Urine sample No.	Treatment	U + S[1] (ml)	Time (min.)	$\frac{U+S}{min.}$	Diuresis (ml/min.)
9'10		A					
9'27	1						
9'30			Inulin infusion started. Rate: 4 ml per minute.				
9'35			250 ml water perorally.				
9'50	2						
9'59		B					
10'06	3						
10'12		C	250 ml water perorally.	150	13.0	11.54	3.8
10'14	4						
10'27		D		252	15.0	16.80	10.1
10'30	5						
10'37,5		E		242	10.5	23.05	13.5
10'39	6						
10'50			Glucose infusion started. Rate: 4 ml per minute.				
10'54	7						
11'01		F		315	23.5	13.40	9.1
11'03			250 ml water perorally.				
11'05	8						
11'09		G		120	8.0	15.00	2.5
11'13	9						
11'18,5		H		129	9.5	13.58	3.1
11'23	10						
11'28		I		170	9.5	17.89	7.4
11'30			Glucose infusion continued. Rate: 6 ml per minute.				
11'31	11						
11'41		J		225	13.0	17.31	9.6
11'44	12						
11'50		K		195	9.0	21.67	10.6
11'53	13						
12'02		L		210	12.0	17.50	9.2
12'05	14		Glucose infusion continued. Rate: 8 ml per minute.				
12'12		M		173	10.0	17.30	7.3
12'16	15						
12'24		N		205	12.0	17.08	8.8
12'26	16						

[1] The term (U + S) indicates the volume of the combined urine and wash water from the clearance period in question.

is practically constant, comprising 2 to 8 clearance periods. This will, to some degree, eliminate errors induced by incomplete or irregular emptying of the bladder.

A typical calculation record is given in Table III.

Fig. 1 shows the relationship between glucose reabsorption (TG) and tubular load. Tubular reabsorption is given as fractional saturation, TG/TmG and tubular load is similarly given as load/TmG. This way of presentation allows individuals with different values of GFR (load=GFR · plasma glucose) and different TmG to be included on the same graph, cf. Fig. 3.

Clearance period No.	Inulin in plasma (mg per 100 ml)	Inulin in urine (mg per 100 ml)	$\frac{U + S}{min.}$ [1] (ml)	Inulin excreted (mg/min.)	Inulin clearance (uncorr.)	Inulin clearance per 1.73 sq.m.	Glucose in plasma (mg per 100 ml)	Glucose in urine (mg per 100 ml)	Glucose excreted (uncorr., mg/min.)	Glucose excreted per 1.73 sq.m. (mg/min.)	Glucose clearance per 1.73 sq.m.	Glucose filtered per 1.73 sq.m.	TG [2] per 1.73 sq.m.	TmG	$\frac{TG}{TmG}$	$\frac{Load}{TmG}$
1	32.8	344.0	11.54	39.70	121.0	138	70	0	0	0	0	97	97		0.37	0.37
2	35.2	221.8	16.80	37.26	105.9	121	76	0	0	0	0	92	92		0.36	0.36
3	37.0	159.8	23.05	36.83	99.5	113	82	0	0	0	0	93	93		0.36	0.36
4	39.0	265.1	13.40	35.52	91.1	104	105	0	0	0	0	109	109		0.42	0.42
5	41.8	299.9	15.00	44.99	107.6	123	165	0	0	0	0	203	203		0.78	0.78
6	43.4	345.9	13.58	46.97	108.2	123	193	22.2	3.01	3.4	2	237	234		0.90	0.92
7	44.6	288.6	17.89	51.63	115.8	132	219	70.2	12.56	14.3	7	289	275		1.06	1.12
8	45.6	271.7	17.31	47.03	103.1	117	263	277.4	48.03	54.7	21	308	253		0.98	1.19
9	45.8	229.4	21.67	49.71	108.5	124	341	517.0	112.03	127.6	37	423	295	259	1.14	1.63
10	45.6	260.4	17.50	45.57	99.9	114	378	951.3	166.48	189.6	50	431	241		0.93	1.66
11	45.2	349.1	17.30	43.09	95.3	109	427	1147.3	198.48	226.1	53	465	239		0.92	1.80
12	44.5	267.9	17.08	45.76	102.8	117	474	1575.9	269.16	306.6	65	555	248		0.96	2.14

[1] The term (U + S) indicates the volume of the combined urine and wash water. [2] TG is the tubular reabsorption of glucose.

Fig. 1. "Glucose titration curve" for subject L. C. (non-pregnant).

Fig. 2. Glucose clearance curve for subject L. J. (renal diabetes).

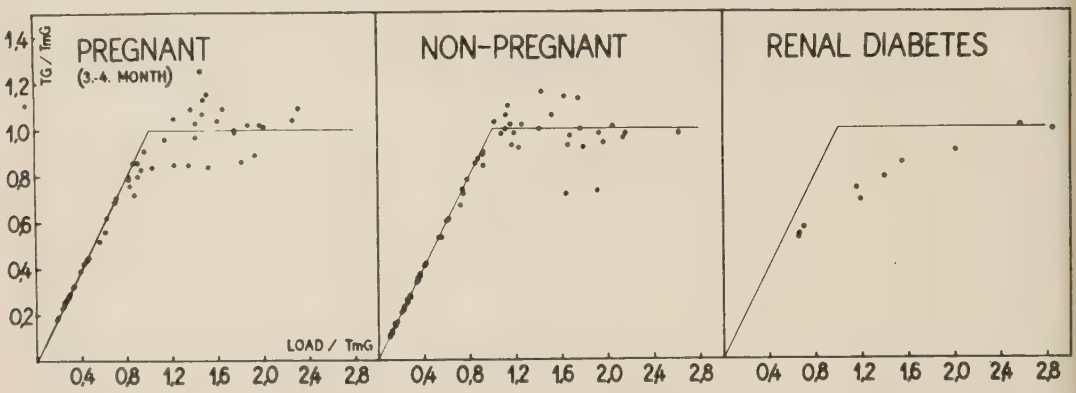

Fig. 3. Mass plottings for pregnant and non-pregnant women, and a single plotting for a renal diabetic, giving the relationship between glucose reabsorption and tubular load.

The renal threshold for glucose is determined by extrapolation on the glucose clearance curve, cf. Fig. 2, to the point of intersection with the abscissa.

The tubular threshold load, *i. e.* the lowest tubular load just giving glycosuria, is calculated as the product of the threshold concentration of glucose in blood in mg per ml and the pre-load glomerular filtration rate.

All calculations are performed *without* correction for errors in urine sampling, and all *with* correction to 1.73 sq. m. body surface.

RESULTS

The results of the investigation are seen in Table IV. Glomerular filtration rate (GFR) is found to be 116 ± 11.3 ml per minute in the non-pregnant state and 152 ± 13.9 ml per minute in the 3rd—4th month of normal pregnancy. The difference between the two groups is significant ($P < 0.01$).

The renal threshold for glucose is significantly decreased in the 3rd—4th month of pregnancy, being 155 ± 17.0 mg per 100 ml blood plasma as compared with the 194 ± 6.5 mg per 100 ml in the non-pregnant state ($P < 0.01$).

The maximal tubular reabsorption of glucose (TmG) is found to be of the same size in the two groups, namely 353 ± 66 mg per minute in early pregnancy and 331 ± 74 mg per minute in the non-pregnant cases.

The two persons examined during late pregnancy have an average GFR of 149 ml per minute, a mean renal threshold of 188 mg per 100 ml, and a TmG averaging 403 mg per minute. The threshold load is within normal for one of them, but considerably elevated for the other one.

None of the persons in early pregnancy have fasting glycosuria. The woman in the 9th month of pregnancy (No. 12) has slightly increased amounts of reducing substances in the urine.

No significant difference in fasting blood sugar level is observed between the pregnant women (67 ± 9.2 mg per 100 ml) and the non-pregnant (65 ± 14.7 mg per 100 ml).

A single person with renal diabetes (No. 6), cf. Table I, has been examined in her 1st month of pregnancy. The GFR found, 132 ml per minute, is within the normal

Table IV. *Inulin and glucose data from the persons investigated.*

on	Physical state	GFR ml/min. per 1.73 sq.m.	Fasting blood sugar mg per 100 ml	Threshold blood sugar mg per 100 ml	Threshold load mg/min.	TmG mg/min.	TmG [1] threshold mg per 100 ml
	Non-pregnant	112	73	195	218	308	265
		131	78	205	269	350	225
		103	48	190	196	449	405
		124	76	190	236	259	210
		110	50	190	209	289	260
S. D.		116±11.3	65±14.7	194±6.5	226±28.3	331±74	273±77
	Renal diabetes (preg. 1st month)	132	68	40	53	134	250
	Pregnant (3rd—4th month)	163	74	160	261	289	175
		168	65	155	260	375	290
		138	56	145	200	296	195
		138	58	135	186	354	280
		152	81	180	274	451	240
S. D.		152±13.9	67±10.6	155±17.0	236±11.7	353±66	236±16
	Pregnant (7th—9th month)	139	73	145	202	278	230
		158	62	230	363	527	310
		149	67	188	282	403	270

e term "TmG threshold" indicates the blood sugar level at which TmG is reached.

nge, cf. Table IV. With a normal blood gar level (68 mg per 100 ml) a pronounced ycosuria is found. The renal threshold, cf. g. 2, is calculated to be 40 mg per 100 ml, ith a corresponding threshold load of 53 mg r minute. TmG is found to be 134 mg per inute, being, like threshold and threshold ad, strongly diminished. A distinct fasting ycosuria was found.

DISCUSSION

The results indicate that the probable cause the diminished threshold is an increased FR and not a change in tubular function, nce the tubular load just causing incipient ucuresis is the same in pregnant and non-egnant women. The TmG values found in rly pregnancy do not diverge from those und in the non-pregnant state. As to late egnancy, the person in the 9th month of gestation has a TmG within the range of the normal group, while TmG in a person in the 7th month of pregnancy is somewhat higher. The findings do not indicate that TmG is changed during pregnancy.

Robertson & Gray (1953) have determined glucose reabsorption twice during pregnancy in a person with renal diabetes. In early pregnancy they find a reabsorption maximum of 810 mg per minute, in late pregnancy 116 mg per minute, and 6 months after delivery 225 mg per minute. The very high TmG value in early pregnancy seems peculiar. The finding is not supported by the present investigation either on normal pregnant persons or on a pregnant renal diabetic, and the observation of Robertson & Gray can hardly be said to be typical of pregnancy.

Donato & Turchetti (1955) present data indicating that TmG is diminished in early pregnancy, rising to elevated values near term. Their TmG of the 3rd—4th month of pregnancy is 272 ± 27 mg per minute and the corresponding figures from the 7th—9th month and for the non-pregnant state (puerperium) are 421 ± 59 mg per minute and 348 ± 25 mg per minute respectively. Only the values given for late pregnancy differ significantly from the TmG values in a larger material of normal women, inulin clearance being used for GFR determination (Smith, 1943). The TmG values of non-pregnant state found by the Italian authors are in accordance with those presented in this paper. The reason why they find an elevation of TmG in late pregnancy may presumably be explained by their finding of an increase in GFR during the second half of pregnancy, using thiosulphate clearance for the determination. This observation does not agree with measurements based on inulin clearance which show elevated GFR values in the first part of pregnancy, approaching normal or even becoming normal in the last two months of gestation, cf. Dill, Isenhour, Cadden & Schaffer (1942), Welsh, Wellen & Taylor (1942), Bonsnes & Lange (1950), Bucht (1951), and Page, Glendening, Dignam & Harper (1954).

The renal tubular function may be changed without influencing the maximal reabsorptive capacity. Such a phenomenon will express itself in a change of shape of the "glucose titration curve" of Homer Smith (1951). A demand for higher load in order to reach TmG will mean a flattening of the curve on the lines of what is seen in certain cases of renal diabetes as presented by Reubi (1954), cf. Fig. 3. The present material affords no basis for the assumption of a change of that kind.

In the present investigation no convincing change in GFR has been observed in connection with increasing glucose loads, cf. Klopp, Young & Taylor (1945), and Dempster, Eggleton & Shuster (1956).

The glycosuria occasionally appearing in pregnancy and first described by Blot (1856) may be connected with the low threshold caused by the increased glomerular filtration, but this is probably not the full explanation, and further research in this field is in progress.

SUMMARY AND CONCLUSIONS

1. The renal threshold and tubular reabsorption of glucose have been investigated in 5 normal, non-pregnant women, 5 normal women in the 3rd—4th month and 2 normal women in the 7th—9th month of pregnancy, as well as in one pregnant woman (1st month) with renal diabetes.

2. In the 3rd—4th month of pregnancy the renal threshold for glucose is decreased corresponding to an elevation of glomerular filtration rate, indicating that the tubular threshold load is unchanged.

3. No difference in tubular reabsorptive capacity between the pregnant and the non-pregnant state has been found.

ACKNOWLEDGMENTS

The present study has been aided by a grant from King Christian X Foundation.

The author is indebted to Professor M. Ingerslev, M. D., the Department of Obstetrics and Gynecology, Aarhus University, Professor V. Aalkjær, M. D., Surgical Department K, Aarhus Municipal Hospital, and Professor G. Nørby, M. D., Marselisborg Hospital, Aarhus, for supplying patients from their departments for the investigation.

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ON THE EQUILIBRATION OF CARBON MONOXIDE BETWEEN HUMAN MATERNAL AND FETAL CIRCULATION *IN VIVO*

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(Received for publication May 23, 1958)

The total amount of hemoglobin of the body can be measured by the alveolar CO method (Sjöstrand, 1948b). A known amount of CO is administered in a closed respiratory system, and after 30 min., necessary for equilibration, the increase of alveolar CO tension is inversely proportional to the total body hemoglobin (Sjöstrand, 1948b). In a pregnant woman, a small part of the administered CO should, *a priori*, be taken up by the fetal hemoglobin, and the maternal total hemoglobin therefore be overestimated. It has been reported (Stucki, 1946) that, after equilibration, the relative fetal COHb concentration (COHb percentage) may be one and a half times as high as the maternal. If such were generally the case, and if equilibration between mother and fetus occurs within 30 min., the overestimation of the amount of maternal hemoglobin might be as high as 10—12 per cent at the end of pregnancy, and would therefore be of practical importance (cf. Gemzell, Robbe & Ström, 1957).

The rate of CO transport from mother to fetus, and the respective final concentrations, have therefore been studied. A preliminary report has been given (Gemzell, Robbe & Ström, 1957).

PROCEDURES AND METHODS

Procedure I

In 14 cases blood samples were taken immediately after delivery from a maternal arm vein and from the umbilical vein (arterialised fetal blood). The Hb concentration and COHb percentage were estimated. The fetal O_2Hb percentage was also measured. The deliveries were spontaneous and normal. No CO, extra O_2, N_2O or chloroform were administered. No signs of fetal distress were noted. The mothers were non-smokers. The lengths of pregnancy, the durations of the labor period and the birth weights were within the normal range. The appearances of the placentae were normal.

Procedure II

In 22 cases, between 22 hours and 12 minutes prior to delivery, CO was administered to the mothers; 27.4 ml CO (STPD) was added to the closed O_2-containing respiratory system into which the mother breathed. This is exactly the same as in a normal determination of total Hb by the CO method. After the administration of CO, the mothers usually breathed in the closed system for 30 min., unless delivery came earlier; they then breathed room air, thus giving off CO slowly. Leakage during the equilibration procedure was difficult to avoid during the strong pains or bearingdown near delivery.

Blood samples were usually taken repeatedly before and after delivery. These, and fetal umbilical vein blood, were analysed as in I above. The length of the pregnancies, duration of labor, birth weights

placentae were within usual limits; the mothers e non-smokers. No extra O_2, N_2O or chloro- n were given, but in one case, because of atening fetal asphyxia, delivery was by low for- , with ether anesthesia. The remaining 21 deli- es were spontaneous, and there were no signs fetal asphyxia.

Procedure III

Blood samples were taken and analyzed as in cedure I in 7 cases where abdominal caesarean ion (medical termination) was performed in the to 24th week of pregnancy. The operations e performed under spinal anestesia, and no N_2O other anesthetic was given before the uterine ion. In these cases the mother breathed in the ed respiratory system containing O_2 till the very. The fetus was extracted about 30 min. r 27.4 ml of CO had been administered to the her. Six of these women were non-smokers.

Methods

The O_2 content and capacity of blood were deter- ed in the manometric van Slyke apparatus. The oglobin concentration was determined spectro- tometrically as O_2Hb in alkaline solution. The content and capacity were analyzed using both rvath & Roughton's method (1942), and Linder- m & Sjöstrand's (1956), as further described by derholm & Söderström (1957) and Linderholm, strand & Söderström (1958). The COHb per- tage in the mother's blood was also estimated irectly by the alveolar method (Sjöstrand, 1948 b), using an empirically found proportionality stant (Carlsten, Holmgren, Linroth, Sjöstrand Ström, 1954).

Horvath & Roughton's method (H. & R.) gave sistently higher values for COHb percentage n the alveolar method (alv.) or Linderholm & strand's method (L. & S.). Thus, in procedure I mean maternal COHb percentage (alv.) was 5 per cent in 14 cases but the H. & R. values e 1.88 per cent COHb, *i. e.* 1.23 ± 0.19 per cent her, while the L. & S. method gave approxi- tely similar (0.07 ± 0.05 per cent higher) values he alveolar method in 8 cases; in 20 maternal or al blood samples the mean COHb percentage (L. & S.) was 0.97 per cent but the H. & R. values were 1.16 ± 0.19 per cent higher. With procedure II the mean maternal COHb percentage (alv.) was 3.58 per cent in 8 cases but the H. & R. method gave 0.87 ± 0.25 per cent higher values, while in 3 maternal or fetal blood samples where the mean L. & S. value was 2.85 per cent, the H. & R. value was 1.34 per cent higher. The CO capacity according to H. & R. was 1.44 ± 0.016 ml CO/g Hb in 41 samples of maternal blood and 1.43 ± 0.015 in 37 samples of child's blood, being significantly higher than the accepted value of 1.34—1.36. The CO capacity according to L. & S. was 1.34 ml CO/g Hb in 6 samples of maternal blood. The method of H. & R. in the present investigation therefore gave slightly too high values for CO capacity, and also gave COHb percentage values in the low range which were approximately 1.2—1.3 per cent COHb higher than the L. & S. values. This difference is probably due in part to the formation of a relatively constant extra fraction of CO during the procedure of the H. & R. method (cf. Carlsten *et al.*, 1954, who found a similar, although smaller, difference within the low range of COHb concentrations), but some other systematic deviation cannot be ruled out for certain. The methodological difference remained constant throughout the investigation period, however, and as the present results depend on differences in COHb concentration rather than absolute values the H. & R. values were used in the final calculations.

RESULTS

Natural ratio between fetal and maternal COHb percentage

When there had been no *exogenous* administration of CO, the fetal COHb percentage was higher than the maternal value (Table I). The ratio of fetal to maternal COHb percentage was 1.18 in 14 cases analyzed by Horvath & Roughton's method (probably in our hands carrying a systematic error leading to too low ratios, see under Methods), and 1.57 in 9 of these cases analyzed by Linderholm & Sjöstrand's method.

Table I. *Carboxyhemoglobin percentage in mother's and child's blood immediately after normal delivery. No CO administered. Number of cases (n), mean (M), and standard deviation (S. D.). Methods of Horvath & Roughton (H. & R.), Linderholm & Sjöstrand (L. & S.), and alveolar method of Sjöstrand (alv.).*

	COHb per cent, mother			COHb per cent, child		COHb per cent ratio child/mother	
Method	H. & R.	L. & S.	alv.	H. & R.	L. & S.	H. & R.	L. & S.
n	14	9	8	14	9	14	9
M	2.02	0.73	0.65	2.25	1.13	1.18	1.57
S. D.	0.76	0.23	0.20	0.78	0.42	0.43	0.33

It is probable that a value of approximately 1.5—1.6 for the spontaneous fetal/maternal ratio is representative. The O_2Hb percentage in the umbilical vein (fetal arterialized blood) was found in 37 cases to be 42.7 per cent (average value, S. D. = 20.5 per cent) independent of whether CO was administered or not, which is a relatively low value (in contrast to Beer, Bartels & Raczowski, 1955; Rooth & Sjöstedt, 1955 and others, but in confirmation of Romney *et al.*, 1955). When umbilical venous blood samples are taken in the absence of labor (caesarean section), normal values for oxygen saturation are reported to be about 50—60 per cent in the 40th week, and progressively lower at higher fetal ages (Walker & Turnbull, 1953).

Fetal and maternal COHb percentage after CO administration

After CO administration to the mother, the maternal COHb percentage is expected to rise rapidly, usually attaining an approximate steady state within 10—15 min. (cf. Forbes, Sargent & Roughton, 1945; Sjöstrand, 1948 b; Hallberg, 1955). The child's COHb percentage, on the other hand, was found to rise slowly (Table II, Fig. 1). In an average of 6 cases, where the maternal equilibration time for CO with a closed system was 12—37 min. (average 24 min.), the child's COHb percentage (H. & R. method) was 2.21 per cent, being only 0.03 per cent above the average naturally occurring value (Table I), while the mother's COHb percentage was 1.97 per cent higher than the average naturally occurring value. If it is assumed that the total amount of hemoglobin is 700 g in the mother (Gemzell *et al.*, 1957) and at most 50 g in the child (Mollison, Veall & Cutbush, 1950; Karlberg & Lind, 1955) including fetal blood left in the placenta (Brody, 1953; Fuchs, 1953), the maternal hemoglobin in these 6 cases would have taken up 18.5 ml CO and the child's hemoglobin 0.02 ml CO out of the 27.4 ml administered. Of the rest of the 27.4 ml CO, small amounts remain in the respiratory system or are taken up by maternal myoglobin; larger amounts leak outwards during the equilibration procedure, and there are also errors in the determination of COHb percentage. In the two cases in Table II where the blood analyses were made by the L. & S. method (24 min. equilibration time), a similar calculation indicates 21.8 ml CO to be taken up by the maternal hemoglobin and 0.28 ml CO by the child's hemoglobin. The

b l e II. *Carboxyhemoglobin percentage in mother's and child's blood, and oxy-noglobin percentage in child's arterialized blood, immediately after normal delivery in ases. Administration of 27.4 ml CO to mother in closed respiratory system 12—37 min. (average 24 min.) before delivery. Symbols as in Table I.*

	COHb per cent, mother		COHb per cent, child		COHb per cent ratio child/mother		O_2 Hb per cent child
od	H. & R.	L. & S.	H. & R.	L. & S.	H. & R.	L. & S.	van Slyke
	6	2	6	2	6	2	6
	3.85	2.92	2.21	1.31	0.59	0.45	53
ge	2.63—4.76	2.78—3.05	1.40—3.34	1.16—1.46	0.36—0.84		22—78

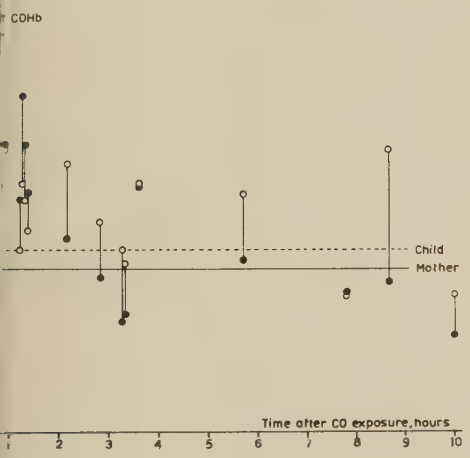

Fig. 1. Carboxyhemoglobin percentage of maternal blood (filled circles) and fetal blood (open circles), determined by the H. R. method immediately after normal delivery in 22 cases. At varying times before delivery (abscissa) 27.4 ml of CO had been administered in a closed respiratory system to the mother, and allowed to equilibrate for about 30 min. Full horisontal line indicates average COHb percentage by the H. & R. method in maternal blood, dashed line that in fetal blood, when no exogenous CO has been administered. Each pair of determinations (mother and child) has been united by a vertical line.

a b l e III. *Carboxyhemoglobin percentage in mother's blood and fetal blood (16—24 eks of fetal age) immediately after abdominal caesarean section in 7 cases of legal ortion. Administration of 27.4 ml CO to mother in closed respiratory system 20— min. (average 29 min.) before uterine section. Fetal hemoglobin concentration 7.0—13.0 g/100 ml. Symbols as in Table I.*

	COHb per cent, mother		COHb per cent, child		COHb per cent ratio	
ethod	H. & R.	L. & S.	H. & R.	L. & S.	H. & R.	L. & S.
n	3	4	3	4	3	4
M	4.64	4.00	3.56	2.77	0.81	0.59

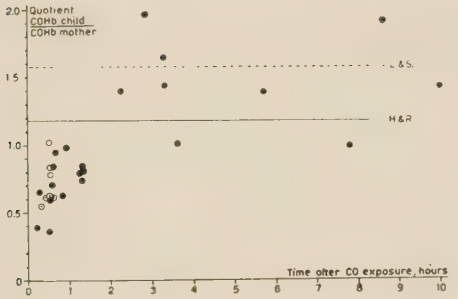

Fig. 2. Fetal/maternal ratio of COHb percentages immediately after normal delivery in 22 cases (filled circles, H. & R. method) and after uterine section in 7 cases of medical termination (open circles for method H. & R., open dotted circles for method L.&S.) At varying times before delivery (abscissa), 27.4 ml of CO had been administered to the mother. The naturally occurring ratio (without CO administration) with H. & R. method for COHb percentage determination is 1.18 ± 0.11 (indicated by horisontal full line), with L. & S. method 1.57 ± 0.11 (indicated by horisontal dashed line).

slow fetal uptake of CO from the maternal circulation results in an initial decrease in the fetal/maternal COHb percentage ratio, as shown by Fig. 1 and 2. This later returns to values above unity, as occurs in cases without CO administration. A similar result was obtained in the medical-termination cases (procedure III), as shown by Table III and Fig. 2.

DISCUSSION

The naturally occurring equilibrium ratio of COHb percentage between fetal and maternal blood was found probably to be about 1.5. This equilibrium should be dependent on several factors such as (i) the rate of CO production of the child in relation to the rate of outward transport of CO to the maternal circulation; (ii) the effective CO and O_2 affinities of fetal hemoglobin in relation to maternal hemoglobin; and (iii) the possible gradient of O_2 partial pressure between maternal and arterialized fetal blood in the placenta. Fetal CO production may be expected to occur, as it occurs in the adult (Sjöstrand, 1949, 1951, 1952) but the rate of production is not known. The CO affinity of fetal hemoglobin is not known (for values for the O_2 affinity of fetal hemoglobin, see Darling *et al.*, 1941; Beer *et al.*, 1955, and others). The O_2 partial pressure of fetal arterialized blood is reported to be significantly lower than the maternal O_2 partial pressure in the placental veins when direct measurements are made (Romney *et al.*, 1955); also indirect computations indicate the presence of a definite gradient (Beer *et al.*, 1955), which would influence the CO equilibrium (cf. Roughton & Darling, 1944). Lack of adequate knowledge of the first two of the three above-mentioned factors, however, prevents an assessment of their relative importance for the fetal/maternal COHb ratio. A production of CO in the child with a slow maternal transfer, a definite maternal/fetal gradient of O_2 partial pressure, and a CO affinity of fetal hemoglobin normally proportional to the O_2 affinity would tend to give higher fetal than maternal values for COHb percentage, as was actually found to occur.

The rate of transport of CO from maternal to fetal blood was found to be slow, with average values at the present maternal blood pCO of 0.02—0.3 ml CO per 24 min. during the initial period of equilibrium. If a transport rate of 0.1 ml CO per min. is accepted the following estimates may be suggested with a maternal blood flow through the placenta of the order of 0.5 l per min. (Assali *et al.*, 1953, Romney *et al.*, 1955), the ma

al arterio-venous CO difference in the nta would amount to such low values as nd 20 microl/l, as compared to a CO ent in maternal blood under these cir- stance of about 6000 microl/l (at 4 per COHb and at a CO capacity of 150). The slow maternal/fetal transfer rate O should depend on the extremely low ma portion of CO in relation to the oglobin portion, and to the kinetics of the dissociation from hemoglobin (Roughton, 5; see also Page, 1957).

he present results indicate that when the amount of hemoglobin of a pregnant an is determined by the alveolar CO hod, using 30 min. equilibration time, fetal uptake of CO causes only a small ematic overestimation, which usually is igible when compared to the non-system- error of the method (4 per cent in a le determination). At term, when the restimation is largest, it would only unt to 1—2 per cent or less.

SUMMARY

Measurements of COHb percentages in ernal and fetal blood immediately after mal delivery show a probable fetal/ma- al ratio of about 1.5—1.6 when the her has not been exposed to exogenous . If exogenous CO is administered to the her before delivery, fetal uptake of CO rs slowly, so that after 30 min. of CO ilibration the fetal/maternal COHb per- age ratio decreases to about 0.5—0.6, rising to values well above unity. Within nin. of CO equilibration the fetal circula- has taken up only 1—2 per cent or less he amount of CO taken up by the ma- al circulation. This result suggests that, in a pregnant woman, the overestimation of the maternal total amount of hemoglobin by the alveolar CO method which is due to fetal CO uptake should usually be negligible.

ACKNOWLEDGEMENT

This investigation has been supported by a grant from "Stiftelsen Terese och Johan Anderssons Minne" to one of the authors (G. S.).

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PROTEIN-BOUND CARBOHYDRATES IN HUMAN FETAL SERUM

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(Received for publication May 28, 1958)

a previous study (Bergstrand & Czar,) concerned with the serum proteins of human fetus a new protein fraction was nstrated by paper electrophoresis. The re of this component, situated between lbumin and the α_1-globulin in the paper , is not known. The possibility that this ein fraction could be related to fetuin ovine fetal serum, first demonstrated by ersen (1944), was briefly discussed in previous communication. Fetuin migrates the α-globulins and has been shown to mucoprotein (Deutsch, 1954).

was thus thought of interest to study the ohydrate content of the different protein ions of human fetal serum including the nown component. No such investigation s to have been published previously.

MATERIAL AND METHODS

e fetuses were obtained from legal abortions d out by caesarian section. The mothers were od physical health. Blood was taken from the s by heart puncture immediately after the tion. From the mothers, blood was drawn at me time from a cubital vein. The fetal sera concentrated to about a third of the original e by evaporation under reduced pressure at temperature.

ra from 10 mothers and 10 fetuses ranging gth from 16 cm to 26 cm were examined.

The total protein content

of the serum was determined by the biuret method.

Total protein-bound carbohydrates

were determined according to Goa (1955) with the modifications given by Nilsson & Åberg (1956). The serum proteins were precipitated by phosphotungstic acid in 2 N hydrochloric acid. The precipitate was dissolved in sodium hydroxide after centrifugating and washing once in 99 per cent ethyl alcohol. Total amounts of carbohydrate were determined with Dische's carbazol reaction. Standard solutions of glucose were treated in parallel and the protein-bound carbohydrates were thus read as glucose. Technical details are given in the paper by Nilsson (1958).

Separation of the serum protein fractions

was made by paper electrophoresis. The technical details are given in a previous communication (Bergstrand & Czar, 1957). The proteins were stained with Ponceau red and the concentration of the different fractions was measured by spectrophotometry after elution of each fraction.

The electrophoretic protein-bound carbohydrate pattern

was determined (Goa, 1955) from an unstained paper strip run simultaneously with the strip used for determination of the protein fractions. The former paper was cut according to the protein pattern of the latter. The cuts were eluted with a solution of saline and sodium hydroxide. After filtration the filtrate was precipitated with phosphotungstic acid

Table I. *Per cent of total carbohydrates in the different protein fractions of fetal serum. ("X" = unknown protein fraction).*

No.	Fetal length in cm	Alb.	X	α_1	α_2	β	γ
1	16	11.6	11.6	21.8	19.4	17.0	19.4
2	17	10.2	12.4	8.4	15.6	4.0	48.2
3	17.5	14.2	7.1	14.2	(43.0)?	14.2	7.1
4	19	29.2	23.5	40.8	0	0	6.6
5	20	21.6	11.8	16.3	14.7	20.0	16.6
6	21.5	20.0	13.5	10.0	20.0	20.0	16.5
7	22	27.2	16.9	11.8	6.7	15.3	22.6
8	23	29.3	22.0	16.9	12.3	14.6	4.9
9	23.5	12.3	9.7	9.7	22.1	31.6	14.6
10	26	9.9	30.8	11.6	13.2	17.3	17.0
Mean		18.6	15.9	13.8	15.4	15.4	17.4

Table II. *Per cent of total carbohydrates in the different protein fractions of maternal serum.*

No.	Alb.	α_1	α_2	β	γ
1	20.6	23.5	20.6	14.8	20.
2	-	-	-	-	-
3	4.5	13.6	45.4	4.5	31.
4	15.0	10.0	40.0	15.0	20.
5	13.4	26.6	20.2	10.0	29.
6	30.1	7.6	15.5	23.4	23.
7	16.7	19.1	14.3	30.9	19.
8	10.8	14.2	17.8	32.2	25.
9	19.9	17.6	35.4	11.1	16.
10	33.4	3.8	26.9	22.1	14.
Mean	18.3	15.1	26.2	18.2	22.

Table III. *Per cent carbohydrates in the different protein fractions of fetal serum. ("X" = unknown protein fraction. PCh = protein-bound carbohydrate).*

No.	Fetal length in cm	Alb.	X	α_1	α_2	β	γ	Total protein in g per 100 ml serum	Total PCh in mg per 100 ml serum	Per cent carbohydrates in total serum proteins
1	16	0.3	1.9	8.6	9.0	7.0	7.2	2.1	37.0	1.8
2	17	0.1	1.3	2.3	3.3	0.9	10.0	3.2	32.5	1.0
3	17.5	0.5	1.6	10.5	(26.8)?	6.2	4.4	1.8	43.5	2.4
4	19	0.6	3.2	12.0	0	0	2.1	2.5	35.1	1.4
5	20	0.5	2.2	8.7	7.9	9.4	5.6	2.0	37.5	1.9
6	21.5	0.3	2.2	3.7	6.5	4.7	3.3	2.1	26.0	1.2
7	22	0.7	3.6	5.3	3.7	10.7	12.0	2.6	49.0	1.9
8	23	0.4	3.0	7.3	3.4	3.6	1.1	3.6	39.0	1.1
9	23.5	0.3	1.5	6.3	10.7	13.7	4.1	2.6	39.0	1.5
10	26	0.2	2.9	3.3	3.1	3.9	2.9	3.0	33.5	1.1
Mean		0.4	2.3	6.8	5.3	6.0	5.3	2.6	37.2	1.5

and centrifugated. The precipitate was washed with ethyl alcohol and dissolved in sodium hydroxide. The carbohydrates were determined with Dische's carbazol reaction and read as glucose. Further details are given in the paper of Nilsson (1958).

RESULTS AND DISCUSSION

The results are given in Tables I—IV.

The total amount of protein-bound carbohydrates in fetal serum appears to be considerably lower than in serum of adults. Th values given in the literature for adult serur are admittedly rather variable but such lo values as found in the present investigatio have not been reported. According to Go (1955) mean values of different autho vary between 102 and 158 mg per 100 m serum with a range of 73 to 192 mg pe 100 ml. The highest values are those obtaine

Table IV. *Per cent carbohydrates in the different protein fractions of maternal serum. (PCh=protein-bound carbohydrate).*

	Alb.	α_1	α_2	β	γ	Total protein in g per 100 ml serum	Total PCh in mg per 100 ml serum	Per cent carbohydrates in total serum proteins
	0.7	16.4	6.7	5.3	3.4	6.9	157	2.3
	-	-	-	-	-	6.7	170	2.5
	0.3	7.6	18.3	1.3	9.5	5.9	209	3.5
	0.7	2.4	5.1	2.7	2.1	6.9	176	2.6
	0.7	29.0	12,7	3.9	6.3	5.5	196	3.6
	1.2	2.2	5.8	7.7	4.6	6.4	165	2.6
	0.5	27.5	5.8	9.8	2.6	6.2	130	2.1
	0.4	4.8	18.3	10.6	4.9	7.2	176	2.4
	1.1	21.4	16.7	2.7	2.3	7.3	230	3.2
	1.8	1.5	7.0	5.3	2.9	6.3	188	3.0
n	0.8	12.5	10.7	5.5	4.3	6.5	180	2.8

Goa's own investigation. It is also of in-st to compare the total amount of protein-nd carbohydrates found in fetal serum in serum of newborns. Sohar, Bossak, ng & Adlersberg (1956) found the mean ıe of total protein-bound carbohydrates in l blood to be 69.3 mg per 100 ml serum Seibert, Atno & Campbell (1947) also orted lower values than in adults. A few rminations have been made in premature ınts and the values found are less than mg per 100 ml serum (Crocco & Coletta, 4). It is well known that total serum tein of the fetus increases during gesta-ı and judging from the few available ures, it seems very likely that the protein-nd carbohydrates also rise during this iod of life. When the amounts of protein-nd carbohydrate are expressed as per t of total protein the values of fetal serum lower than those given for normal adults those found in the mothers of the present estigation. This is in contrast to the find-s in infants, who according to Kellner & lhof (1955) have significantly higher levels of protein-bound carbohydrates (expressed as per cent of total protein) than adults.

During gestation the maternal serum shows an increased level of protein-bound carbohydrates (Südhof & Kellner, 1957). The mean value for total protein-bound carbohydrates in maternal serum found in the present investigation is somewhat higher than the mean value for normal serum given by Goa (1955). The difference is, however, not significant.

As seen from the tables the values for the carbohydrate content of the electrophoretically separated protein fractions show rather great variations. This may be due to the technical difficulties of the method, and as the number of sera examined is small it is necessary to draw conclusions from the figures very cautiously. Goa (1955) found that the protein-bound carbohydrates of normal serum are roughly equally distributed between the different protein fractions. The results of the present investigation point in the same direction and there seems to be little dif-

ference in this respect between maternal and fetal sera. The values are also of the same order of magnitude as those given by Goa (1955) for normal serum.

Goa (1955) found rather great variations in the amount of carbohydrate expressed as per cent of protein in the various fractions of normal sera. The figures (Table IV) for maternal sera presented here also vary considerably but the mean values especially for the α-globulin fractions appear higher than those given by Goa.

The amount of carbohydrate in the different globulin fractions (Table II) is roughly the same in all the fetal sera. Albumin and the unknown protein component ("substance X") appear to have a lower content of carbohydrate than the globulins.

The difference between the mean value for "substance X" and the mean values for the different globulin fractions are, with the exception of the γ-globulin, significant at least on a 2 per cent level.

The carbohydrate content of the unknown protein component is of special interest. As already mentioned, Deutsch (1954) has shown that fetuin of fetal bovine serum has the properties of a mucoprotein. He found that various fetuin samples contained approximately 8 per cent glucosamine and 9.5 per cent mannose. Fetuin thus appears to have a much higher content of carbohydrates than the unknown protein fraction of human fetal serum. These observations do not favor the conception that the unknown protein is related to fetuin.

SUMMARY

The total protein-bound carbohydrate content in serum of 10 mothers and 10 fetuses was determined with the method described by Goa (1955).

The amount of carbohydrates in the different protein fractions separated by paper electrophoresis was determined with the same method.

The mean value for total protein-bound carbohydrates in maternal serum was found to be 180 mg per 100 ml serum.

Fetal serum shows a lower content of protein-bound carbohydrates expressed in mg per 100 ml serum and as per cent of total protein than adult serum. The mean values found were 37 mg per 100 ml serum and 1.5 per cent respectively.

The carbohydrate content of the different protein fractions of maternal and fetal serum are presented in tables.

The carbohydrate content of a previously demonstrated unknown protein fraction of fetal serum is relatively low. This seems not to favor the conception that this component is related to fetuin of bovine fetal serum.

ACKNOWLEDGEMENT

The technical assistance of Miss Lola Berkow is gratefully acknowledged.

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Scandinav. J. Clin. & Lab. Investigation 384—389, 10, 1958.

THE ESTIMATION OF FIBRINOLYTIC COMPONENTS BY MEANS OF THE LYSIS TIME METHOD

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(Received for publication June 10, 1958)

The assay of lytic activity by measuring the lysis time of standard fibrin clots has been widely used in investigations on the fibrinolytic system, and was described especially by Christensen (1949). This method has been used here for the estimation of activator, proactivator, and streptokinase, and it was surprising that a much higher degree of accuracy than ordinarily obtained could be reached through a few modifications of the procedure.

The functions of the components are shown in the following schemes which give a brief summary of our present knowledge of the mechanism of the streptokinase induced fibrinolysis:

streptokinase + proactivator $\rightleftharpoons$ activator

$$\text{plasminogen} \xrightarrow{\text{activator}} \text{plasmin}$$

$$\text{fibrin} \xrightarrow{\text{plasmin}} \text{split products}$$

It should be emphasized that there is some doubt about the nature of the proactivator since Sherry & Alkjaersig (1956) presume identity between this factor and plasminogen, and suppose the activator to be a complex of streptokinase and plasminogen or plasmin.

The use of the lysis time method for measuring activator, proactivator, and streptokinase is based upon the availability of the following preparations.

1. Purified bovine fibrinogen which contains large amounts of plasminogen but none of the other components mentioned in the above schemes.

2. Purified preparations of streptokinase in which plasmin inhibitors are the sole interfering contaminants.

3. Human plasma which contains large amounts of proactivator. The interfering contaminants are plasmin inhibitors and plasminogen.

Briefly, the method consists of mixing activator, thrombin and bovine fibrinogen at zero time and measuring the time elapsed until redissolution of the clot formed. The lytic effect of the activator in this system is due to the transformation of the bovine plasminogen into plasmin which dissolves the fibrin (Astrup & Permin, 1947). Proactivator and streptokinase are measured by incubating the component concerned with an excess of the other component and determining the lytic effect of the activator formed. In all cases lysis times are converted into concentrations of the components concerned by interpolation on standard curves.

MATERIALS AND METHODS

Buffers: 1. 0.05 M-sodium diethyl barbiturate fer pH 7.75 to which was added NaCl to give inal ionic strength of 0.15. This buffer will be rred to as veronal buffer.

2. Veronal buffer to which 200 mg of gelatin 100 ml was added (0.2 %). This buffer will be rred to as veronal-gelatin buffer.

Streptokinase: "Varidase" was kindly placed at disposal by the Lederle Laboratories Division, nerican Cyanamid Company. The units are those ted on the packages and determined according Christensen (1949).

Proactivator: Citrated human plasma, or human sma acetone powder prepared as described assen, 1958).

Bovine fibrinogen with a purity of 70—90 % was pared according to Astrup & Müllertz (1952). was dissolved in veronal buffer and immediately ore use diluted to 0.25 % with the same buffer.

Thrombin: A preparation of bovine thrombin taining about 20 000 NIH units (National Institute of Health) per g was kindly placed at my posal by Løvens kemiske Fabrik, Copenhagen. lutions containing 2 mg per ml veronal-gelatin ffer were prepared daily.

Manometric determination of plasmin: Plasmin s measured by the method previously reported assen, 1958) using benzoyl-L-arginine ethylester AEe) as substrate.

Estimation of activator: Generally the activator s contained in concentrated solutions which were uted with veronal-gelatin buffer before the lysis ne determinations. The activator concentrations the diluted solutions never exceeded 50 units/ml the case of streptokinase and 0.5 % (v/v) in the e of plasma. Gelatin was added to the buffer avoid adsorption of streptokinase to glass surces described below.

To 1 ml of the diluted solution of activator 0.1 of thrombin was added and the mixture was inbated for exactly 3 min. at 37°. (Due to the ility of activator it is important to use a well ined time of incubation.) 1 ml of a 0.25 % solun of bovine fibrinogen was now added and mixed shaking the test tube for 4—5 sec. The fibrino- solution was previously incubated for 5—10 n. at 37°, the exact time of incubation being of minor importance. About 30 sec. after mixing a clot was formed, and after about 2 min. small air bubbles could be observed in the clot. When the clot lysed the bubbles rose to the surface, and the lysis time was defined as the interval of time between the addition of fibrinogen and the point of time when the bubbles from the bottom of the test-tube had reached half-way up to the surface.

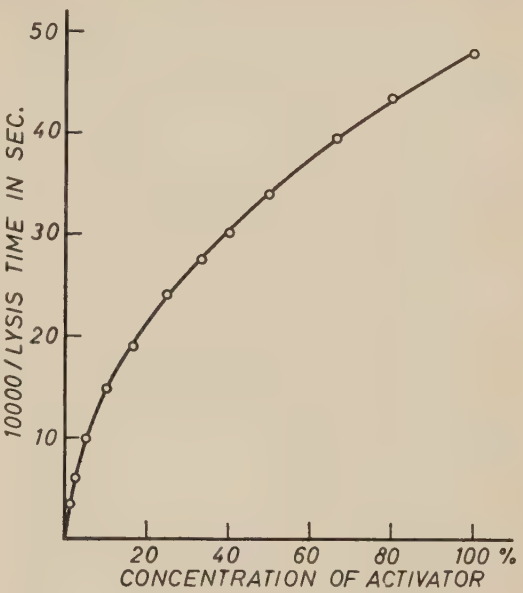

Fig. 1. A standard curve for activator concentration. Preparation of stock solution of activator: 100 μl plasma were added to 100 μl of a solution containing 1000 units of streptokinase. After standing for 20 min. at room temperature the mixture was diluted in ice bath with 10 ml veronal-gelatin buffer.

Lysis times were converted into concentrations of activator by interpolation on a standard curve which was obtained by estimating the lysis times of serial dilutions of a given preparation of activator (Fig. 1). The curves obtained with different batches of fibrinogen differed in shape and position, and a new curve was therefore made every day. That the preparations of fibrinogen contained no proactivator was controlled by incubating 1 ml of streptokinase (50 units/ml) with thrombin and fibrinogen as described. Batches of fibrinogen which lysed within 24 h. were discarded.

Estimation of proactivator and streptokinase: The component concerned was incubated at room temperature together with an excess of the other component. The amounts used for the estimations were: *Streptokinase:* 100 µl of a solution containing 75 mg plasma acetone powder per ml and 50 µl of the sample in which the concentration of streptokinase should not exceed 5000 units per ml. *Proactivator:* 50 µl of a solution containing 10,000 units of streptokinase per ml were used together with 50 µl of the sample in which the concentration of proactivator should not exceed that of undiluted plasma. After incubation for 25 min. at room temperature (22°) the mixtures were diluted with 10 ml veronal-gelatin buffer, and the lytic effect of these solutions was then measured, as described, for the estimation of activator. Lysis times were converted into concentrations by interpolation on standard curves obtained under exactly the same conditions as in the experiments. These standard curves were generally not much different from the standard curves used for the estimation of activator. This is in accordance with Müllertz (1955) who found that the amounts of activator formed in mixtures of streptokinase and proactivator were proportional to the concentration of one component, provided that the other component was present in excess.

INVESTIGATIONS ON THE METHOD

Standard deviation: The standard deviation of a single estimation of activator was found to be 0.7 % of the mean value (lysis time = 7 min., number of estimations = 12). The deviation seemed to be nearly the same for all lysis times between 3½ and 60 min.

The influence of inhibitors: Plasma and the preparation of streptokinase both contain large amounts of plasmin inhibitors. To determine the effect of these inhibitors lysis times were measured with a constant concentration of activator and using fibrinogen containing varying concentrations of plasma or streptokinase (Table 1). The table shows inhibitors to be ineffective within the concentration range usually employed.

Table I. *The inhibitory effect of plasma and streptokinase in the lysis time method.*

Lysis times were determined as described with a constant concentration of activator and using fibrinogen containing the amounts of plasma or streptokinase stated below.

Plasma. Activator solution: 50 µl of plasma (excess) + 125 units of streptokinase dissolved in 50 µl veronal-gelatin buffer were incubated for 20 min. at 22° and then diluted with 10 ml buffer.

µl plasma/ml fibrinogen	lysis time in min.	relative activity
0	6^{55}	41.8
6.25*	6^{54}	42.0
12.5	6^{55}	41.8
25	7^{07}	39.0
50	7^{25}	35.0
100	7^{52}	30.0

Streptokinase. Activator solution: 50 µl plasma + 1000 units of streptokinase (excess) dissolved in 100 µl veronal-gelatin buffer were incubated for 20 min. at 22° and then diluted with 20 ml buffer.

units streptokinase/ml fibrinogen	lysis time in min.	relative activity
0	6^{15}	54.8
25*	6^{15}	54.8
50	6^{14}	55.0
100	6^{16}	54.6
200	6^{20}	53.6
400	6^{28}	51.4

* The maximum concentration of plasma or streptokinase otherwise used in the method.

The influence of human plasmin: The lytic effect of the small amounts of human plasmin which inevitably contaminate the preparation of activator was determined. A preparation of human plasmin free of activator was made by heating streptokinase-activated human globulin to 100° at pH 2 as described by Müllertz (1955). The following solutions were then prepared: 1. A solution of activator containing plasmin made by mixing plasma with excess of streptokinase (10,000 units per ml of plasma). 2. A solution containing the same concentration of

ma and the amount of plasmin necessary ive the same BAEe esterase *activity* as tion 1. This solution contained no activ-, but the same concentration of plasmin olution 1 (Lassen, 1958). The two solu-s were then diluted to give a final con-ration of plasma equal to 0.5 % of un-ted plasma, and lysis times were meas-l as described. The solution containing activator lysed in about 4 min. and that of activator in 85 minutes. After a her 200-fold dilution, solution 1 gave a s time of 80 min. Comparisons of the effect of the two preparations in lower centrations gave even larger relative dif-nces. Essentially the same results have obtained with acetone dried preparations roactivator. This implies that under the dition of the above experiment the lytic ct of the human plasmin can be neglected. en high concentrations of proactivator low concentrations of streptokinase are d such neglect is scarcely justified, since amount of plasmin obtainable was found e nearly independent of the concentration treptokinase (Christensen, 1945, Troll & rry, 1955), while the concentration of vator is proportional to the concentration treptokinase (Müllertz, 1955).

The concentration of fibrinogen and mbin: Fig. 2 shows the lysis times ob-ed with serial dilutions of activator using different concentrations of fibrinogen. slight dependence on the concentration ibrinogen is probably due to the simul-eous change in the concentration of the minogen. Only very small changes in s times were observed when the concen-ion of thrombin was 0.5 or twice the centration elsewhere used.

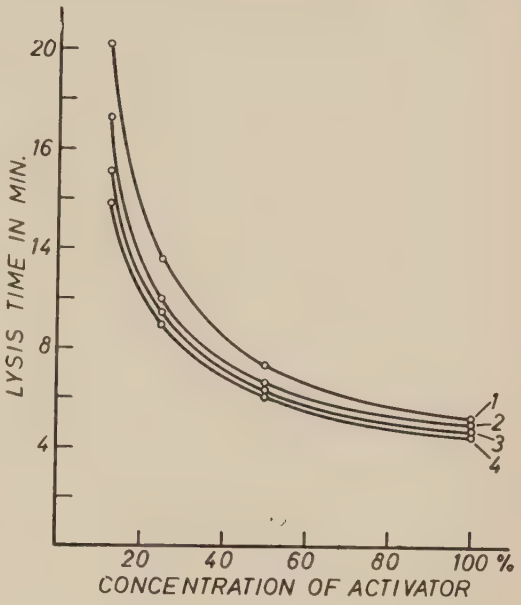

Fig. 2. Relationship between lysis time and activator concentration using four different concentrations of fibrinogen. Abscissa: concentration of activator. Ordinate: lysis time in minutes. The final concentrations of fibrinogen were: Curve 1: 0.3 %, curve 2: 0.2 %, curve 3: 0.15 %, and curve 4: 0.1 %.

The adsorption of streptokinase on glass: Adsorption on glass surfaces was found to introduce errors in the determinations of streptokinase when veronal buffer was used as the solvent. This adsorption was demonstrated by stirring 2 ml veronal buffer containing 4000 units of streptokinase for 30 min. at room temperature with 500 mg of finely powdered glass (size unknown), and then centrifuging. Less than 1 % of the original amount of streptokinase remained in the supernatant.

Several compounds were tested for their ability to prevent this supposed adsorption of streptokinase, but only proteins were found to be effective (Table II). With casein and

Table II. *Adsorption of streptokinase on glass surfaces.*

2 ml of veronal buffer containing 2000 units of streptokinase per ml plus different proteins in various concentrations were tested for streptokinase before and after stirring for 30 minutes with 500 mg of finely powdered glass (size unknown).

Protein	Concentration (per cent)	Relative activity: Before stirring with glass powder	Relative activity: After stirring with glass powder
None	-	100	1
Gelatin	1	111	113
»	0.5	111	113
»	0.2	109	112
»	0.1	110	107
»	0.05	109	81
Casein	1	108	107
Bovine Serum Albumin	1	107	71
Haemoglobin	1	120	86
Witte peptone	1	150	83

gelatin present no significant adsorption occurred, but gelatin was preferred since casein is insoluble in acid solutions. The lower initial activity of the protein free solution was supposed to be caused by adsorption of streptokinase to the inner surface of the 50 μl Carlsberg pipette used in the estimations. Detailed investigations on the special effect of Witte peptone will be published later. The adsorbed streptokinase could be completely eluted by stirring for 30 minutes with veronal buffer containing 1 % gelatin showing that the adsorption is reversible.

The use of gelatin buffer is necessary for a correct estimation of streptokinase, as the following experiment shows: A solution (150 μl) containing 2000 units of streptokinase/ml of veronal buffer were poured into a 50 ml conical flask, and the solution was allowed to cover the whole inside of the flask.

Table III. *The effect of gelatin in the lysis time determinations.*

50 μl samples, containing 2000 units of streptokinase per ml, were mixed with 50 μl of a solution containing 75 mg of acetone dried plasma protein per ml. After 30 minutes 10 ml veronal buffer were added, and lysis times estimated as described. Furthermore, the solutions contained no (—) or 0.2 per cent gelatin (+). A solution of activator was used for the standard curve.

	0.2 per cent gelatin present in: The solution of streptokinase	The buffer used for the dilution	The solution of fibrinogen	Relative activity in per cent
1	+	+	—	100
2	+	—	—	94
3	—	—	—	86
4	—	—	+	78

Determinations of streptokinase revealed that 60 % of the total amount was adsorbed to the glass surface.

The effect of using 0.2 % gelatin is shown in Table III where the inhibitory effect of gelatin itself is shown in experiments number 3 and 4. The slight difference indicates a much higher affinity of plasmin for fibrin than gelatin.

DISCUSSION

By means of the lysis time method it was found possible to obtain very accurate estimations of the plasminogen activator. The influence of possible interfering contaminants has been investigated, and it was found that plasmin inhibitors are ineffective within the concentration range used in the method, and that the lytic effect of the contaminating human plasmin is at least nearly insignificant.

The method was found useful too for the determination of proactivator and strepto-

se, which are the precursors of the plas-
ogen activator. However, later experi-
ts (not published) seem to indicate that
e are some fundamental difficulties in
estimations of the precursors. These dif-
lties probably arise from the fact that the
unt of activator which is generated by
ing streptokinase and proactivator de-
ds on more than one component in each
he two preparations. This, in connection
h the impossibility of obtaining the same
portions between the "active components"
he experimental solutions and the solu-
s used for the standard curve, introduces
rs into the values obtained by interpola-
of the experimental results on the stan-
d curves. (The expression "active com-
ent" denotes all components which in-
nce the formation of activator, and does
imply that all such components are ne-
sary for the occurence of the reaction.)
Streptokinase was found to be strongly
orbed on glass surfaces. The adsorption
glass surfaces of purified proteins in di-
d solutions has been described before
g. catalase, Svendsen, 1953; testicular
luronidase, Rasmussen, 1954). Total elu-
n of streptokinase can be achieved with
ite solutions of gelatin. It is not known if
s is caused by true elution or by a displace-
nt. If the latter is the case, the use of
atin buffers would probably be valuable
other cases where adsorption on glass in-
feres with enzyme measurements.

SUMMARY

. The lysis time method has been devel-
d for the determination of activator, pro-
vator, and streptokinase.

2. Streptokinase was found to be adsorbed on glass surfaces. Complete elution has been accomplished by addition of gelatin to the buffer.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Tage Astrup for his interest and helpful advice. This investigation was made possible by a grant from "Statens almindelige Videnskabsfond", Denmark. It forms part of the investigations on fibrinolysis for which Dr. Astrup receives support from The Josiah Macy, Jr. Foundation, New York, and the National Danish Association against Rheumatic Diseases.

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Scandinav. J. Clin. & Lab. Investigation 390—393, 10, 1958.

THE CORRELATION BETWEEN THE OCCURENCE OF HISTAMINE IN BLOOD AND URINE

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(Received for publication June 9, 1958)

The role of the endogenous histamine in allergic and anaphylactic reactions is yet an unsolved problem chiefly because of the difficulty in finding a reliable method for demonstrating changes in the histamine metabolism in the body. As was shown by Schayer & Cooper (1956) there are several histamine metabolites in man, but methods for their determination, suitable for routine use, are not yet available. At present, therefore, determination of free and conjugated histamine in tissue, blood or urine seems to be the method of choice for routine work. In the blood most of the histamine is associated with the leukocytes (Code & Mitchell, 1957) and it is uncertain if any free histamine is present in plasma. Infusion of histamine intravenously gives rise to an increase of the blood histamine only for a short period (Emmelin, 1951) and assay of histamine in the blood thus reflects the histamine level only at the actual moment when the sample is collected. For illustration of the condition during longer periods determination of histamine in the urine seems to be of greater value. However, a prerequisite for using this method for such a purpose is to ascertain by clear evidence that the determinable histamine in the urine reflects the blood histamine level.

The source of the histamine excreted in the urine has not been definitely shown. Adam (1950) and Adam, Card, Riddell, Roberts & Strong (1954) suggested, that the free histamine normally excreted in the urine derives from the plasma. Lindell & Schayer (1958 a) studied the urinary excretion of free histamine and its metabolites after infusion of C^{14} histamine into the renal artery. They found that "of the C^{14} histamine removed from the blood by the kidneys, part (25 per cent) is excreted in the urine as histamine, part is oxidized to imidazole acetic acid and part is methylated on the ring nitrogen remote from the side-chain to form methylhistamine".

The purpose of this work is to study whether the amount of free histamine excreted in the urine is correlated to the blood level and, thus, might reflect the histamine release in the body.

MATERIAL

Histamine was determined in blood and urine in 28 persons. The urine was collected in 24 hours periods and the corresponding blood samples were taken in the morning. The first group of the material consisted of 5 healthy subjects and 14 patients with increased amounts of histamine in blood and urine. In this group are included cases of myeloid and basophilic leukaemia, carcinoid tumours, Loeffler's syndrome and three cases without

n etiology of the increase in histamine. The est histamine values were obtained in the leu- hia cases.

a second group (Fig. 2) are collected 9 cases evere burns, examined with histamine deter- tions during the first 96 hours after the acci- These cases have been described earlier ke, Dunér, Liljedahl, Pernow, Plantin & ll, 1958).

v. infusions of histamine were done in eight hy subjects.

METHODS

xtraction of histamine from blood and urine performed as previously described (Dunér & ow, 1956, 1958).

those cases in which histamine was admi- red this was done by intravenous infusion of iologic saline solution with a histamine con- ration of 10 μg per ml. The infusion rate was sted so as to cause the least possible discomfort e subject. In all cases, 5 to 10 mg were given e course of 5 hours. Blood samples were taken re and during infusion. The urine was collected g the day of infusion and for 48 hours prior nd 48 hours after the day of infusion.

RESULTS

The correlation between the histamine content of the blood and the urinary excretion of free histamine.

ig. 1 shows the content of free histamine he urine correlated to the blood histamine centration. Since the material includes mal as well as selected pathologic cases, ery wide range of variation was obtained n regard to the content of free histamine he urine (2.2—800 μg per 24 hour ex- ion). This amount is well correlated to amount of histamine in the blood.

he correlation between blood histamine urinary histamine was also clearly illu- ted by the values found in patients with

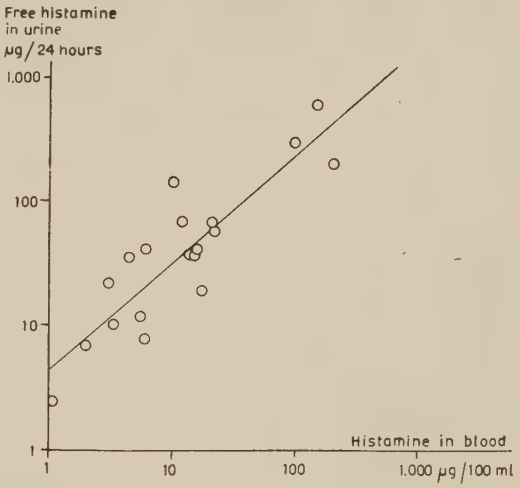

Fig. 1. Urinary excretion of free histamine (μg per 24 hours) in normal and different pathological cases in relation to histamine in whole blood (μg per 100 ml). The regression line equation: $y = 0.865\ X + 0.649$ and coefficient of correlation $(r) = 0.88$.

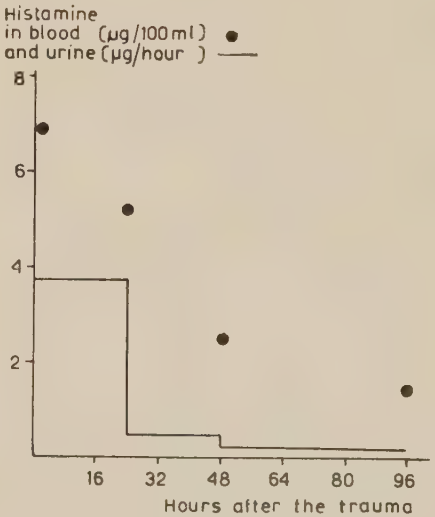

Fig. 2. Histamine in blood and urine in burns (mean of nine cases) during the first 96 hours after the accident.

burns, in whom the histamine content was high in the first 24 hours and thereafter rapidly fell to normal or subnormal values. Fig. 2 shows the mean values of nine patients with severe burns.

2. Intravenous infusion of histamine.

In all the subjects the excretion of both free and conjugated histamine before infusion was within the limits of previously published normal values (Dunér & Pernow, 1956). During the day of infusion it was markedly increased, and calculated on the infused amount of histamine the increase constituted 0.8 to 3.0 per cent, which is in agreement with the values reported by Adam (1950). Similarly, a slightly increased content of histamine in the blood was demonstrated during the infusion. A typical experiment is shown in Table I.

Table I. *Histamine in blood and urine before and after i.v. infusion of 5.0 mg histamine in 630 ml saline, given in the morning on the third day.*

Day	Urine volume in ml	Histamine in urine (μg per 24 hours) free	Histamine in urine (μg per 24 hours) conjugated	Histamine in blood (μg per 100 ml)	
1	650	8.3	29.2	5.8	
2	570	8.9	40.0	4.9	
3	1000	53.3	123.0	7.4	(during infusion)
4	930	8.5	50.0	5.1	
5	800	6.5	43.1		

Percentage of infused histamine excreted as free histamine: 0.9 per cent.

DISCUSSION

The results reported give evidence of a correlation between histamine in blood and urine, which suggest that the histamine occurring in a free state in the urine is removed by filtration from the blood, thus to some extent reflecting the endogenous histamine metabolism in the organism as a whole. Other observations point in the same direction. Bloom, Dunér, Pernow, Winberg & Zetterström (1957) and Brogren, Dunér, Hamrin, Pernow, Theander & Waldenström (in press) have shown that patients with urticaria pigmentosa excrete large amounts of histamine in the urine. One of the patients had a solitary tumour-like skin lesion with abundant mast-cells. After excision of this lesion the excretion of histamine fell quickly to normal values. This fact can only be explained by liberation of histamine from the histamine-rich tumour, which was reflected by a high excretion.

It is not known, if the formation of histamine in the kidney, which has been demonstrated after infusion of histidine into the renal artery (Lindell & Schayer, 1958 b) is also occurring in man. The results collected in Fig. 1 might indicate that, if such a formation exists, it is probably of an insignificant degree.

The conjugated histamine in urine is probably acetylhistamine (Urbach, 1949), absorbed from the intestinal tract. Histamine administered orally is excreted entirely in conjugated form (Urbach 1949, Adam 1950), whereas subcutaneously injected histamine is said to be chiefly excreted in free form (Anrep, Yadi, Barsoum, Smith & Talaat 1944). In our experiments, however both free and conjugated histamine in urine increased after intravenous infusion of histamine. This and results from studies in patients with mastocytosis (Brogren *et al.*, in

ss) give evidence that the conjugated amine in urine can partly derive from ogenous released histamine.

There is a group of pathologic conditions nely some acute manifestations of hyper-sitivity, for instance acute asthmatic at-ks and food allergy where a discrepancy sts between the amount of histamine in od and urine. During these acute stages histamine content of the urine falls to measurable amounts (Dunér & Pernow, 58 b), while the blood histamine level is entially unchanged or raised (Serafini, 48, Rose, Rusted & Fownes, 1950). ether this phenomenon is caused by block-of the excretion, accelerated break-down the kidneys or conjugation of the active tamine is not known.

SUMMARY

There is a good correlation between the tamine content of the blood and the uri-y excretion of free histamine. After in-venous infusion of histamine the urinary cretion of free histamine increases. This rease equals about 0.8 to 3.0 per cent of infused amount.

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HISTAMINE AND LEUKOCYTES IN BLOOD DURING MUSCULAR WORK IN MAN

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The role of histamine has been discussed in connection with the "reactive hyperaemia". Lewis (1927) considered that the vasodilatation occurring in a tissue that had previously been rendered ischaemic would be associated with release of histamine caused by the anoxaemia. Emmelin, Kahlson & Wicksell (1941), on the other hand, were unable to find any increase of the histamine content of the venous blood from an ischaemic limb. In guinea-pigs, which were exposed to hypoxia corresponding to an altitude of 6000 metres for three weeks, there occurred a rise in blood histamine, which reached a maximum after 48 hours (Dunér, Pernow & Tribukait, in press).

In the blood from a contracting muscle, Anrep & Barsoum (1935), in experiments on dogs, found that the histamine content was increased in comparison with blood collected at rest. The increase in the histamine content was proportional to the time and degree of muscular contraction. It was also much greater than the increase produced by only temporary clamping of the artery. On the basis of these results the present investigation was made in an attempt to find out whether in man any change occurs in the histamine content of the blood during muscular work. Since the main part of the histamine that can be demonstrated in blood seems to occur in the leukocytes (Code, 1937 and others), the number of leukocytes was estimated in each blood sample.

METHODS

The material comprises 15 healthy persons, women and men aged from 20 to 40 years, who were subjected to work on a bicycle ergometer. They had a normal physical working capacity, which for women varied between 600 and 800 kpm per minute and for men between 900 and 1200 kpm per minute, according to the criteria for this test recommended by Sjöstrand (1947) and Wahlund (1948).

A polythene catheter for collecting blood samples was inserted into one of the brachial arteries by the method described by Berneus, Carlsten, Holmgren & Seldinger (1954). Blood samples for white-cell count, including differential count, and samples for determination of blood histamine were taken at rest before the test and during muscular work at the maximal load immediately before the termination of the work. The duration of the work was 18—24 minutes.

For white-cell count duplicate samples were taken on each occasion, as was also done for the differential count. The latter was carried out in blood films, and at least 200 cells were counted in each sample.

In ten of the cases 15 ml of blood was taken each time for determination of the histamine content in whole blood. In five of the cases 25 ml of blood was taken, which was immediately centrifuged, and the histamine content could in these

ɛs be determined in plasma and blood cells arately.

The extraction of histamine in whole blood in- tes, in brief outlines, haemolyzation, precipita- of the proteins with acetone, adsorption on an exchanger (Amberlite IRC-50), eluation and ntitative determination on the guinea-pig ileum. ncipally the same method was used for extrac- of histamine in serum and blood cells (Dunér Pernow, 1956 and 1958).

RESULTS

As is seen from Table I the histamine tent of arterial whole blood before muscu- work was on an average 4.2 ± 0.7 μg 100 ml of blood and rose to an average 6.7 ± 0.7 μg per 100 ml of blood during maximal exertion, the difference being istically highly significant ($P = < 0.001$). e white-cell count on the same occasions raged $5\,700 \pm 620$ and $8\,800 \pm 860$ re- ctively, which also represents a significant rease during muscular work ($P = 0.001$). The white-cell differential count able II) showed no significant difference ween the values before and after work.

In those five cases in which the histamine tent was estimated in serum and in blood ls separately, there was a significant in- ase of the histamine content both in the od cells and in the serum (Table III). another series of experiments (7 cases) re was no difference between the amount histamine in arterial and mixed veneous od.

DISCUSSION

During muscular work there was a signi- ant increase in the histamine content of blood. In those cases in which the hista- ne content was determined simultaneously both serum and blood cells there was a tendency to increase in both fractions. It is uncertain whether this increase is primary in both plasma and blood cells or if the rise in the serum is due to a diffusion of hista-

Table I. *Amount of histamine in whole blood and number of white blood cells in 10 healthy subjects before and during muscular work.*

	Histamine μg per 100 ml blood	Number of white blood cells per mm³ blood
Before work	4.2 ± 0.7	$5\,700 \pm 620$
During work	6.7 ± 0.7	$8\,800 \pm 860$

Table II. *Mean values in per cent from differential count of leukocytes in arterial blood in 15 cases of healthy subjects before and during muscular work.*

	Neutrophils, band forms	Neutrophils, segmented forms	Eosinephils	Basophils	Lymphocytes	Monocytes
Before work	1.0	57.0	3.5	0.5	32.5	5.5
During work	1.0	55.0	2.5	0.5	35	6.0

Table III. *Amount of histamine in serum and blood cells and number of white blood cells in arterial blood in 5 healthy subjects before and during muscular work.*

	Histamine μg per 100 ml whole blood		Number of white blood cells per mm³ blood
	in serum	in blood cells	
Before work	1.6 ± 0.3	3.8 ± 0.5	$4\,400 \pm 440$
During work	2.9 ± 0.7	5.6 ± 0.9	$6\,600 \pm 520$

mine from the increased amount of leukocytes. This study gives no answer to the question whether the histamine in serum occurs in a biologically active or in a conjugated form. The extraction procedure is in fact likely to release adsorbed or conjugated histamine. There is supporting evidence for the assumption that histamine may be liberated from the leukocytes, for instance in connection with allergic reactions (Katz & Cohen, 1941) and, hence, the histamine in the leukocytes cannot, as is often done, be considered to be of no biological importance. The function of the leukocytes as conveyors of many important enzymes and other biologically active substances, among them histamine, has become the object of increasing attention (Larsen, 1957 and others). It has also been shown that the leukocytes are actively secernating cells (Richter 1955, Thiery 1957). The physiologic significance of the leukocytosis and the increased content of histamine in blood during work is not clear. However it is not impossible that the dilatation of the capillaries occurring in the working muscle is partly induced by histamine.

SUMMARY

The leukocyte count and the histamine content of the blood have been determined in 15 subjects at rest and during work on a bicycle ergometer. At work the number of leukocytes increased and the histamine content showed an increase both in serum and blood cells. The possible physiologic significance of these observations is discussed.

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RAPID CHANGES IN HUMAN RIGHT HEART BLOOD EMPERATURE AT VARIATIONS IN VENOUS RETURN*

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(Received for publication June 19, 1958)

e temperature in various parts of the shows, under normal conditions, mar- differences even in deep tissues (Hor- Rubin & Foltz 1950, Eichna, Berger, r & Becker 1951). The term "body erature" is discussed in detail by Burton dholm (1955) emphasizing that objec- can be made against any site proposed epresenting an average of deep body erature. The results in the present paper rm this statement and we agree with conclusion of Burton & Edholm "that it better to measure the temperature at the ted site and to consider the phenomena rded in relationship to the temperature e site rather than confuse the issue by g that temperature body temperature". a previous paper (Carlsten & Grimby) it was demonstrated that right heart d temperature in man is fairly constant st, whereas rapid changes in intracardiac erature could be induced at certain tions in the distribution of blood. As heart blood is composed of blood from pheral and central parts of the body, it ed reasonable to expect that shifting in proportion of blood returning to the right le coming on the one hand from tissues warm blood and on the second hand the cooler periphery should be reflected in the temperature of the blood in the right auricle. The present experimental data concerning right heart blood temperature are given with special reference to the amplitude and duration of changes in this temperature induced by pooling blood in the lower limbs.

Supported by a grant from Svenska Nat.för. Tuberkulos och Andra Folksjukdomar.

METHOD AND MATERIAL

The thermoelectric instrument (Ellab, Copenhagen, Type TE 3) and error of methods have been described by Sigroth (1957). The thermocouples are made of stainless steel and constantan. The reference unit is included in a metal block with great thermal capacity. The measuring units were introduced into different vessels, esophagus and rectum. The electromotive force between the two thermocouples is measured on a mirror galvanometer.

The rectal applicator, a rod shaped capsule of metal with the thermocouple adapted at the tip, was inserted to a distance of about 5 cm from the sphincter. A measuring thermocouple at the end of a nylon catheter was introduced into an ordinary esophageal tube and heart catheter, respectively, up to a few mm from the open tip of these. The end of the esophageal tube has been localized about 40 cm from the teeth i. e. in lower esophagus. The effect of swallowed saliva on esophageal temperature has been avoided with the aid of a saliva suck pump. The heart catheter was inserted under fluoroscopic guidance in the routine manner from a cubital vein of the left arm into the centre of the right auricle. The tip of the catheter fitted with the thermocouple was directed towards the inflow of blood from the inferior caval vein.

The subjects were investigated in recumbent position after a period of twenty minutes to allow the temperature to become stable. Occlusion cuffs on the lower limbs with the distal border 15 cm proximal to the patellar base were inflated (i) to 70—80 mm Hg for 5 minutes and after that (ii) to 220 mm Hg. After 5—10 minutes the tourniquets were suddenly deflated from 220 to 0 and pooled blood was released. All the subjects examined, 14 in total, had normal temperature and arterial blood pressure. In two investigations the legs containing pooled blood were immersed for 12 minutes in water of 7° C and after an interval of 60 minutes in water of 44° C whereas in all other experiments the lower limbs were kept in room temperature (about 20° C). Muscular temperature is known to fall upon cooling the skin covering the muscle (Bing, Carlsten & Christiansen, 1945). The room was maintained free from draughts.

The temperature from the right auricle, esophagus and rectum were continuously recorded before, during and after pooling of blood in the legs in 6 healthy volunteers, aged 18—39 years.

We had also the opportunity to measure the temperature of blood leaving the limbs after occlusion in connection with routine venography, in diagnosis of pelvic tumours in 8 patients, performed in the X-ray Department. Bilateral percutaneous catheterization of the femoral vein are always performed in these cases (Bartley 1958). Using these catheters as channels the temperature of the femoral venous blood from both legs could easily be measured simultaneously. An occlusion cuff was applied only to the one leg and inflated in the same procedure as described above. The temperature in the femoral vein of the leg with intact circulation was used as a control.

RESULTS

1. *Blood temperature in the femoral veins.*

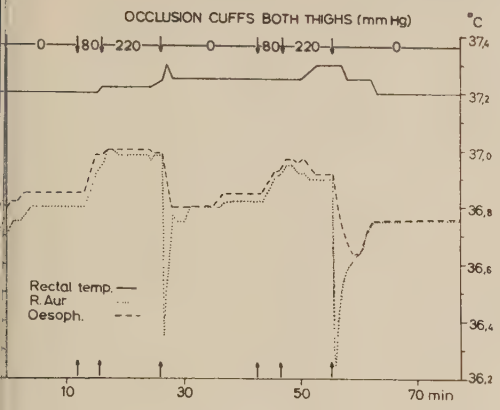
Pooled blood from the one leg was released after arterial occlusion during 5 minutes and a fall in temperature of the blood in the femoral vein of that side was recorded, the maximal fall being 2.3° C, minimal 0.4° C and mean about 1° C. The temperature decrease was maximal 10—25 seconds after the deflation of the tourniquets and after a duration of 2 minutes the preocclusion temperature was restorded. In the contralateral femoral vein, with uninterrupted blood flow, no definite change in the temperature occurred.

As was self-evident and predicted, blood from peripheral tissue with venous and arterial occlusion is cooler than blood from the same tissue at intact circulation. On release of such pooled blood from the legs to the general circulation a lowering of total blood temperature is expected to occur and in proportion to the volume of trapped blood and the duration of occlusion. For obvious reason such variations of the experimental procedures could not be performed on these patients examined by venography for diagnostic purposes.

2. *Right heart blood temperature.*

In the 6 volunteers the temperatures of right auricular blood and lower esophagus were equal at rest in recumbent position whereas rectal temperature was always higher (0.4° C—0.8° C). A representative experiment is demonstrated in figure 1. The temperature in heart and esophagus rose by 0.2° C when venous return from both legs was blocked, presumably because the part of returning venous blood coming from the peripheral "cool" tissues decreased with a relative increase of warm blood from the organs contained inside the cavities. The rectal temperature on the other hand changed very little during this manoeuvre.

When the tourniquets, applied to the bare legs, were promptly deflated to zero the right



1. From above rectal, esophageal and right cular blood temperatures before, during and r pooling blood in the lower limbs. 24 years old male healthy volunteer.

icular blood temperature started to fall er a period of latency of about 5 sec. ig. 1). The maximal fall in heart blood nperature was established 20—40 sec. er the release of circulation in the legs, ereas a fall in lower esophageal tempera- e could be observed after a period of la- cy of about 1 minute and the maximum fall about 1—2 minutes later. In addition the slower reaction in esophageal tempe- ure the total fall in temperature was far s than in the heart. After 5—10 minutes rt and esophageal temperatures returned the preocclusion values. Rectal tempera- e was practically unchanged.

The maximal cooling of right auricular nperature at the procedures described ounted to 0.6° C—0.9° C.

In two of the volunteers the procedure was dified in the respect that the legs including oled blood were not kept in room tempe- ure but were immersed for 12 minutes o cold water (7° C) and after 60 minutes o warm water (44° C). At the moment

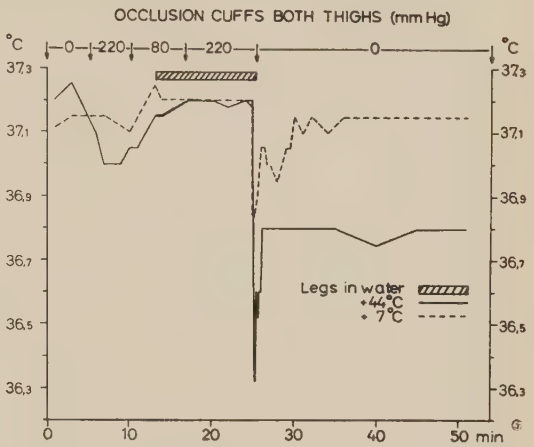


Fig. 2. Right auricular blood temperature in a 19 year old male healthy volunteer in connection with blood pooling in the lower limbs and immersion into cold and after an interval of 1 hour warm water.

of deflation of the tourniquets from 220 mm Hg the lower limbs were again kept horizontally in room temperature. On release of the venous return from the legs the right auricular blood temperature fell more after immersion in warm water than in cold (Fig. 2). The following rise of temperature to normal level occurred as in the previous experiments after 5—10 minutes only after the cold immersion, whereas in the two experiments with warm immersion the right heart blood temperature was subnormal (0.3° C—0.4° C below preocclusion level) during 30 minutes observation time after the deflation of the cuffs.

DISCUSSION

In the study on the function of the right auricle in man, especially at variations in blood distribution and venous return, we found it necessary to control also possible

changes in the temperature of the blood in the right auricle during such experimental procedures. Changes in respect of rate and rhythm of the pacemaker, provoked by rapid variations in the volume of returning blood to the right auricle, might also be modified by changes in blood temperature.

Blood temperature in peripheral vessels shows great fluctuations. The blood acts as a common carrier of heat from one area to another, a function which is incompatible with a constant thermal load (Bazett, 1949).

The rapid fall in the blood temperature of the right auricle after release of pooled blood from the legs demonstrated in the present paper could not be predicted from measurements of the rectal temperature. The lower esophagus, localized close to the heart, should be a better indicator of heart blood temperature and Stupfel & Severinghaus (1956) stress that in these two sites the temperature is practically equal. The present results confirm that esophageal and right heart temperature agree closely but it must be emphasized that this applies only to *steady state*. The esophageal temperature reacts much slower than that of the heart, a fact illustrated by the rapid temperature fall of short duration in the right auricle which was only indicated as a tendency in the esophageal temperature.

The large and rapid fall of blood temperature of the right auricle when pooled blood from the legs was allowed to return to the heart, is interpreted as the result of an absolute increase of cold peripheral blood in the right heart blood which is a mixture of blood from the core and the periphery. The results from temperature measurements in the femoral veins show a cooling of peripheral venous blood at arterial occlusion confirming the conclusion by Bazett, Love, Newton, Eisenberg, Day & Forster (1948) and Scholander & Schevill (1955) and also that there exists an exchange of temperature between arterial and venous blood when the vessels run close to each other.

A more puzzling observation is the protracted fall in right auricular temperature existing at least 30 minutes after release of pooled blood from legs previously immersed in warm water of 44° C. This temperature was 0.3° C—0.4° C lower than before and might be the result of a re-distribution of blood within the body, between the inner organs and the periphery with an increase of blood flow through peripheral tissues especially the skin of the lower limbs after the warm immersion.

The physiological significance of the temperature changes observed is certainly not drastic, but may be of some importance. Bazett *et al.* (1948) have summarized this topic.

Variations in venous return induce changes in right auricular blood temperature. This is indicated firstly by the present observation that the heart temperature increases slightly on venous occlusion of the lower limbs and secondly by unpublished observations (Carlsten & Grimby 1957) with slight fall in esophageal temperature during a few minutes after entering a bath of + 44° C, when accelerated venous flow from the skin and periphery in the direction of the heart might be assumed.

The rectal temperature is constantly higher than that of the right heart blood and lower esophagus, a fact also reported by Horvath *et al.* (1950), and presumably due to the

heat protection in the rectum and per- also due to heat production from bac- metabolism (Pickering, 1958). In the ussion of rectal temperature the obser- ons of Mead & Bonmarito (1949) should onsulted. They found various values in temperature due to variations in tech- e. In the present study the rectal tempe- re was registered with a solid applicator beyond the anus and should represent an value of the intraluminal rectal tem- ture.

conclusion it may be stressed that right cular temperature in man is fairly con- t at steady state but that rapid and pro- nced fall in this temperature can be pro- d with procedures resulting in return of e venous blood volumes from peripheral es. These rapid variations in heart blood perature can only be quantitatively eva- ed from direct measurements in the right t.

SUMMARY

After arterial occlusion during 5 minu- venous blood from the lower limbs is ed by about 1° C.

Pooled blood leaving the lower limbs r venous and arterial occlusion provokes ll in right auricular blood temperature by C—0.9° C. The temperature is restored r 5—10 minutes except after immersion warm water of the limbs including poo- blood. With this procedure right heart d temperature is subnormal for more 30 minutes.

Lower esophageal temperature is a l indicator of right heart temperature in steady state, whereas rapid variations eart temperature is quantitatively badly cted in the esophagus.

4. Rectal temperature is always higher than the temperature of heart and esophagus and shows no, or very slight, changes in variations of the distribution of blood in the body.

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THE INFLUENCE OF CHLOROTHIAZIDE ON THE RENAL FUNCTIONS

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(Received for publication June 25, 1958)

Chlorothiazide, which is a very effective diuretic, has in vitro a strong inhibiting effect on carbonic anhydrase (Beyer, Baer, Russo, Haimbach 1957). It is still under discussion as to what degree there is any such effect in vivo. It may seem that — in the living organism — the effect corresponds more to that which can be obtained from mercurial diuretics, *i. e.* an increased excretion of equivalent amounts of Na and Cl ions. It has been emphasized that the effect on the K excretion by chlorothiazide is negligible. This, however, does not correspond to the clinical experiences (Bayliss, Marrack, Pirkins, Rees & Zilva 1958, and own experiences). Due to the above mentioned facts we considered a close examination of the influence of chlorothiazide on the renal function to be indicated.

MATERIAL AND METHODS

Seven healthy volunteers, 5 men and 2 women were used for the experiments. The chlorothiazide was given by mouth or intravenously in different doses.

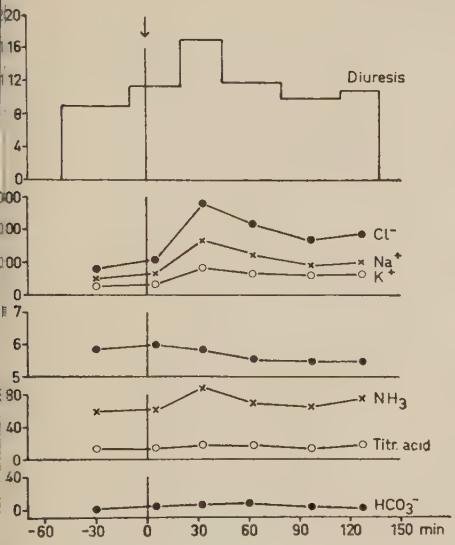
In some of the experiments ammonium chloride was given before and/or during the experiment in order to stimulate the kidney's ability to acidify the urine. It was assumed that the alkalinization of the urine, which the preparation is expected to exert on account of its carbonic anhydrase inhibition, would be more easily established in this way.

Four experiments with peroral administration were performed, all on the same subject (J. B. I, II, III, IV). In each one of these experiments ammonium chloride was given; 0.1 g per kg body weight the day before, and 2—4 g in repeated doses during the course of the experiments.

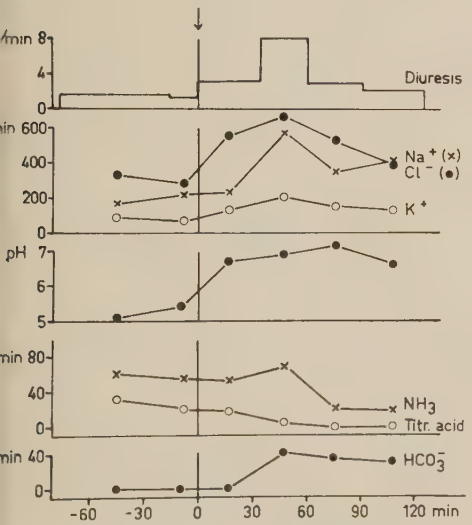
In experiments J. B. I and J. B. IV the subject drank 1—1½ liter of water the morning before the test. In experiments J. B. II and J. B. III he had nothing to drink before, but in J. B. III 5 IU pitressin tannate in oil was given intramuscularly on the afternoon of the previous day.

The six experiments with intravenous administration of chlorothiazide were carried out on different subjects, four of whom were given 500 mg and two 1000 mg in single injections. Two drank 1—1½ liter of water the morning before the test. To one of these, and to one non-prehydrated subject ammonium chloride was given (0.1 mg per kg body weight).

In those cases where chlorothiazide was given intravenously PAH-, inulin- and endogenous creatinine clearance was performed. Catheterization of the renal vein was performed in three of the tests in order to obtain an accurate determination of the renal plasma flow. Further, the diuresis, the excretion of Na, K, Cl, ammonia, titratable acid and bicarbonate were followed. The pH and the osmolarity of the urine were also determined. The clearance technique and the methods for determination of Na, K and Cl have been described in previous papers (Bucht 1951, Ek 1955). Ammonia was determined by the method of Conway (1947) and pH with a glass electrode at room temperature. The titratable acidity was determined by electrometric titration to pH 7.4. Total CO_2 was determined in accordance with Van Slyke and Neill (Peters & Van Slyke 1932). The osmolarity was determined with a thermistor device built in this laboratory by Dr. J. Rosensweig, Belfast.



1. J. B. I. Chlorothiazide given by mouth. ne subject was water-loaded before the test.



2. J. B. II. Chlorothiazide given by mouth. No water-loading beforehand.

RESULTS

hree of the four experiments where rothiazide was given by mouth are illu- ted in figures (Fig. 1—3). The results of

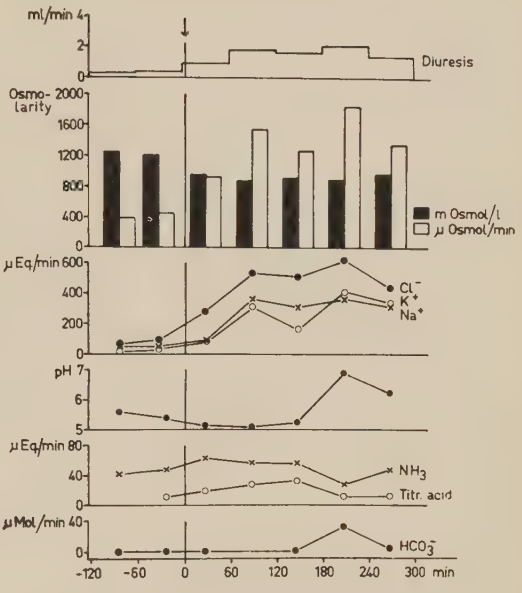


Fig. 3. J. B. III. Chlorothiazide by mouth. Pitressin was given beforehand. The increase of K-excretion came much earlier than the signs of carboanhydrase inhibition.

the fourth experiment largely corresponds to the test J. B. I (Fig. 1). The results from those experiments where chlorothiazide was given intravenously are accounted for in Fig. 4—6 and in Tables 1 and 2. As there was no significant quantitative difference in effect whether the intravenous dose was 500 or 1000 mg the mean values were calculated for all these tests together. Because no principal difference was observed in electrolyte excretion whether ammonium chloride was given or not, the results are presented together in the figures and the tables.

1. Diuresis increased in all cases immediately after chlorothiazide had been given. The increase appeared earlier when chlorothiazide had been given intravenously. When the subjects were hydrated before the experiments the increase of diuresis was always smaller when counted in per cent of the pre-

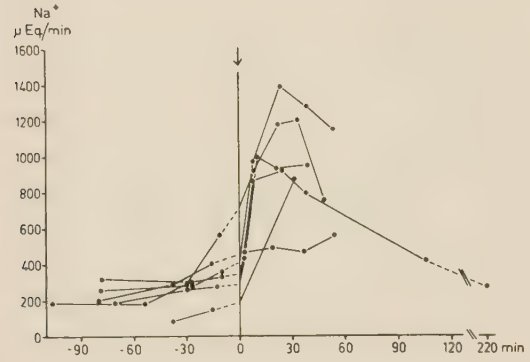


Fig. 4. The sodium excretion in six cases before and after intravenous injection of chlorothiazide.

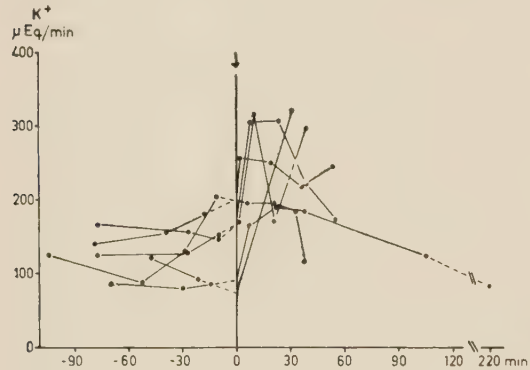


Fig. 5. The potassium excretion in six cases before and after intravenous injection of chlorothiazide.

administration value. In experiment J. B. IV (Fig. 3) where 1 ml pitressin was given on the day before the test, only a very small increase of the diuresis appeared.

2. Inulin-, PAH- and endogenous creatinine showed no clear-cut change (Table I). The light decrease of the renal PAH-extraction possibly depended upon the fact that chlorothiazide interferes with PAH at the chemical determination. Nor did the renal plasma flow show any distinct change.

3. The osmolar excretion per minute showed a marked increase in all experiments.

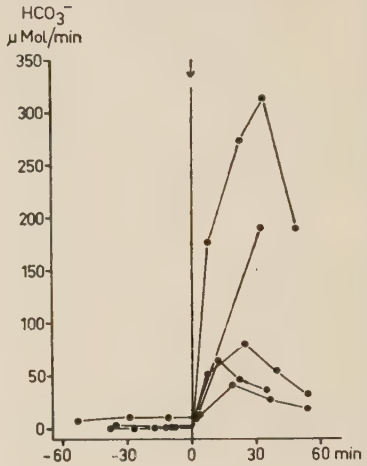


Fig. 6. The excretion of bicarbonate in five cases before and after intravenous injection of chlorothiazide.

T a b l e I. *The effect of intravenously injected chlorothiazide on clearance values, PAH-extraction and renal plasma flow. Weighed mean values corrected to 1.73 m² body surface area.*

	C_{In}	C_{Cr}	C_{PAH}	Extraction PAH	RPF	FF %
n	6	6	6	3	3	6
mean before C	97	92	491	88.6	590	20.8
mean 1st hour after C	103	92	517	84.3	591	19.3

4. The sodium excretion increased heavily in all experiments (Fig. 1—4). The largest increase was from 311 µEq/min. before, to 1162 µEq/min. during the hour after the intravenous injection.

5. The Cl-excretion increased parallelly with the Na-excretion. The largest values were from 366 µEq/min. before to 1190 after.

Table II. *The effect of intravenously injected chlorothiazide on electrolyte excretion and pH. Weighed mean values corrected to 1.73 m² body surface area.*

	μOsmol/ min.	Na^+	K^+	Cl^-	HCO_3^-	NH_4^+	titr. acid	pH
n	5	6	6	6	5	6	5	5
mean before C	1203	248	120	323	2.1	42	45	5.23
mean 1st hour after C	2341	826	224	899	101	24	27	6.73

5. The K-excretion increased in all tests espective of whether any signs of carbonic ydrase inhibition appeared or not (Fig. -3, 5). The largest increase was from 96 296 μEq/min. in one test where chloro- azide was given intravenously.

7. In all experiments where chlorothiazide s given intravenously pH increased. In st of the cases pH rose from about 5 to and in one case from 6.5 to 8.5. The ex- tion of ammonium decreased in these es by 25—50 μEq/min. The titratable dity decreased by 5—50 μEq/min. The cretion of bicarbonate increased in all ex- riments (Fig. 6). At the tests J. B. II and (Fig. 2 and 3) where chlorothiazide was en by mouth the results corresponded to se, obtained by intravenous injections. At test J. B. III the effect did not appear til after 3 hours. At tests J. B. I and IV ere the subject was given 1—1½ liter of ter to drink before the test, no signs of bonic anhydrase inhibition were found ig. 1).

DISCUSSION

When, in December 1956, we started the ical tests with chlorothiazide given by uth to patients with heart incompensation, were not sure how much of the drug was sorbed from the intestinal tract. The main son for our doubts was that we saw large fluctuations in the effect, which could not be completely explained by the extent of the incompensation or by any possible disturbances in the electrolyte balance. We therefore decided to compare the effect of this drug when given by mouth with the effect when it was given intravenously.

The four tests which have been performed with supply, by mouth, of the drug to a normal man did not show any univocal results concerning carbonic anhydrase inhibition. In two of these experiments where the diuresis initially was low, we encountered what was considered as an inhibitory effect, i. e. an increase of pH of the urine, an increase of the excretion of sodium bicarbonate and a decrease of the excretion of ammonium and titratable acidity. In two tests with high diuresis no signs of inhibition were observed, while, on the other hand, the effect on Na- and Cl-excretion was marked. The K-excretion increased in all cases irrespective of whether any signs of carbonic anhydrase inhibition were apparent or not. It is possible that the difference in effect if the patient had had water before the test or not could have depended upon ingested water load disturbing the resorption of the drug, with a lower blood concentration as a consequence.

When pitressin effect was at hand the diuresis increased only slightly, but, nevertheless, the excretion of electrolytes increased

greatly. Thus, it seems probable that the diuretical effect of chlorothiazide does not depend on a pitressin inhibition nor on a decreased capacity of the tubules to reabsorb water. Because the effect of the drug appears very quickly after intravenous injection a depressed secretion of ADH is not probable. The most probable explanation of the increase of the diuresis in the pitressin experiment seems to be an increased osmotic load on the distal tubules.

To eliminate irregularities in the effect caused by variations in the resorption, six experiments were performed with the drug given intravenously. After these relatively large injections signs of carbonic anhydrase inhibition were always observed together with an increased K-excretion. The increase was not larger in the cases that received the larger dose. Even with the smaller dose (500 mg) the maximal effect was obtained on both salt excretion and carbonic anhydrase activity. This latter effect seemed to be less outspoken.

Our results show that when treating edema with chlorothiazide one has to be attentive to the risk of hypopotassiemia even when doses are given that do not have any inhibitive effect on the carbonic anhydrase. This agrees with the clinical experiences. To ten patients with mitral lesion, who were not treated surgically, as little as 250 mg chlorothiazide was given twice daily. When checked by one of us (Bucht) signs of hypopotassiemia were found in nine of these cases.

The effect of intravenous supply seems to be practically immediate. This is not without importance in acute circulation disturbances, for instance pulmonary edema. (Chlorothiazide for intravenous use is not yet obtainable on the market.)

We are grateful to Messrs. Merck Sharp & Dohme for generous supplies of Chlorothiazide.

SUMMARY

The effect of chlorothiazide on the renal functions has been examined in ten experiments on seven healthy volunteers. In most experiments it produced effects which strongly indicated an inhibitory effect on the renal carbonic anhydrase, i. e. an increase of the pH of the urine and an increased excretion of sodium and carbonate ions. The potassium excretion was increased whether there were any signs of carbonic anhydrase inhibition or not. The effect is immediate after intravenous supply. When giving 500 or 1000 mg intravenously the effect seems to be independent of the dose. No effect on the glomerular filtration rate or the renal plasma flow was observed.

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A COLORIMETRIC METHOD OF DETERMINING UNESTERIFIED FATTY ACIDS IN PLASMA

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(Received for publication July 8, 1958)

he presence in the serum of unesterified acids was demonstrated over a hundred s ago by Hoppe-Seyler. The observation verified by von Szent-Giörgyi & Tomin- (1924) among others. Evidence that the er observations had not been laboratory facts was presented by Kendall (1941) Cohn, Hughes & Weare (1947), who d the albumin fraction of plasma to con- unesterified fatty acids. Although ma has been known for so long to con- unesterified fatty acids, their physiologic ortance was not studied in any great il until very recently. These later in- igations have shown the albumin-bound sterified fatty acids to be a form of trans- for fat which is readily available for abolic demands (Dole 1956, Gordon & rkes 1956, Laurell 1956, Spitzer & Mil- 1956, Bierman, Schwartz & Dole 1957, don, Gates & Cherkes 1957, McCalla, es & Gordon 1957, Bragdon & Gordon 8, Gordon & Cherkes 1958, Laurell 8).

arlier methods of determining the un- rified fatty acid content of plasma were d on either weighing (von Szent- gi & Tominaga) or on titration (Stewart White, 1929, Davis 1947, Grossman, n, Becker & Moeller 1954, Dole 1956, don, Cherkes & Gates 1957, Svanborg & Svennerholm 1958). For the titrimetric methods, 1 to 3 ml of plasma were used, the whole volume being necessary for the analysis.

An extremely sensitive method for determining unesterified fatty acids was described by Murkerjee (1956) and Coleman & Middlebrock (1957). It is based on the enrichment of methylene blue between aqueous phase and fat solvent, and is reported by Coleman & Middlebrock to be capable of determining the unesterified fatty acids in 0.2 ml of plasma.

For our purposes it was desirable to evolve a method of estimating the unesterified fatty acids as well as the glycerides, phospholipids, and cholesterol in one and the same lipid extract. The advantages of such a method are obvious when working with small quantities of plasma. We also wanted a method of high specificity for determining long chain fatty acids (more than 14 carbon atoms). Since the procedure was intended for routine analysis, we thought it best that the final determination should be colorimetric.

The principle of the method developed is as follows: Plasma is extracted with chloroform/methanol. After filtration the extract is shaken with acid phosphate in a separatory funnel. The phospholipids are separated on silicic acid and the unesterified fatty acids

on an ion exchanger. After methylation, the unesterified fatty acids are converted to hydroxamic acids which are determined colorimetrically. The final estimation of unesterified fatty acids is worked out to be sensitive to amounts of between 0.5 and 4.0 μEq fatty acid.

METHOD

Solvents and Reagents

Extraction

Methanol and chloroform distilled once in all-glass apparatus.

Chromatography

Distilled chloroform.

Reagent grade silicic powder (Baker), "activated" for 18 hours at 110° C.

Hyflo Supercele commercial grade.

Ion Exchanger Separation

Ion exchanger: Amberlite IRA 400, reagent grade.

Diethyl ether is dried with calcium chloride and distilled over potassium hydroxide.

95 per cent ethanol.

Reagent grade glacial acetic acid (Merck).

Ethanolic ether: diethyl ether containing 5 per cent (v/v) 95 per cent ethanol.

Acid ethanolic ether: ethanolic ether containing 5 per cent (v/v) glacial acetic acid.

Determination of Fatty Acids

Reagent grade methanol (Merck).

Absolute thionyl chloride (Merck) distilled at 74—76° C and used immediately.

Diethyl ether dried over calcium chloride and distilled over potassium hydroxide. It is of great importance to the blank values of the reagents that the ether is free of peroxide.

The reagents for the hydroxamic acid reaction are those suggested by Rapport & Alonzo (1955).

Ethanolic hydroxylamine hydrochloride: a 3 per cent solution of hydroxylamine hydrochloride in 95 per cent ethanol.

Ethanolic sodium hydroxide: a 3 per cent solution of sodium hydroxide in 95 per cent ethanol.

Alkaline hydroxylamine solution: equal volumes of ethanolic hydroxylamine hydrochloride and ethanolic sodium hydroxide are mixed. Filtration after 15 minutes. The mixing and subsequent filtration should be done just before the solution is to be used for the reaction.

Iron reagent: 0.5 g ferric perchlorate (non-yellow, G. Frederick Smith Chemical Company, Columbus, Ohio) is dissolved in 487 ml of 95 per cent ethanol and 13 ml of 70 per cent perchloric acid. This solution is stable for 2 weeks at a temperature of + 4° C.

Test Substances

Reagent grades of the different fatty acids were used. The triglycerides were purified by chromatography on silicic acid and/or recrystallized. The di- and monoglycerides were kindly supplied by Dr. B. Borgström, Lund, Sweden.

Procedure

Sampling: Blood from a cubital vein is collected in ice-chilled centrifugal tubes containing heparin (5 mg heparin to 10 ml blood). Immediate centrifugation and removal of plasma for extraction at + 4° C.

Extraction and Purification of the Fat Extract: 2 ml of plasma are pipetted into a 50 ml volumetric flask containing 16 ml of methanol. Approximately 20 ml of chloroform are added, and the mixture is given a short boil. After cooling, the extract is diluted with chloroform to volume. The extract is filtered through fat-free filter paper. After filtration, 40 ml of the extract are withdrawn and shaken with 10 ml of 0.5 M acid sodium phosphate in a separatory funnel. After partition of the phases overnight, the chloroform phase is drawn off and evaporated on the water bath to approximately 2 ml volume. The phospholipids are separated from this fat extract by chromatography on silicic acid (Borgström 1952 a).

Chromatography: The phospholipids are separated on a chromatographic column (internal diameter 7 mm) containing activated silicic acid (0.5 g) and Hyflo Supercele (0.25 g) which are packed in petroleum ether and washed with chloroform. The fat extract dissolved in approximately 2 ml of chloroform is passed through the column which is then rinsed with 20 ml of chloroform. The phospholipids are adsorbed quantitatively and can be eluted with 10 ml of methanol.

When the phospholipids have been separated, the roform phase is evaporated almost to dryness. extract is then dissolved in approximately 4 ml thanolic ether and the unesterified fatty acids rated.

on Exchanger Separation of Unesterified Fatty ds: Columns with an internal diameter of 7 mm used. Amberlite IRA 400 is regenerated with hydrochloric acid and 1 N sodium hydroxide e. The ion exchanger material in OH-form is hed with distilled water to neutral reaction. roximately 0.5 g of ion exchanger material weight) is packed in wet state on the column. column is then washed with 5 ml of 95 per ethanol and after this with repeated volumes thanolic ether.

The fat extract dissolved in ethanolic ether is passed slowly through the ion exchanger co- n at a rate of roughly 10 drops per minute. column is rinsed twice with 4 ml of ethanolic r. Elution of the fatty acids from the ion ex- nger is done with acid ethanolic ether in such ay that 4 ml is added and the outflow from the mn stopped, after which the ion exchanger is ntained in acid ethanolic ether overnight ughly 18 hours). The eluate is withdrawn and column again eluted twice with 4 ml of acid nolic ether at a drip rate of 10 drops per min- ; this is collected together with the initial eluate ctly into Kjeldahl microflasks. It is important the ion exchanger is never allowed to dry at stage of the elution procedure.

Determination of Fatty Acids: The solvents are porated in vacuo (15 mm Hg) to dryness. The y acids are dissolved in 1 ml of methanol, after ich 0.5 ml of thionyl chloride is added care- y. The solvent and reagent are evaporated in uo and each flask evacuated entirely by aspira- n under reduced pressure (15 mm Hg). 3 ml ether are added to each flask and 0.1 ml of aline hydroxylamine solution. The ether is porated at 62—68° C and the flasks evacuated aspiration under reduced pressure, after which nl of iron reagent is added. The colour is read er 30 minutes at 520 mμ.

Blank and standard solutions are run concur- tly, both for the colour reaction alone and for whole purifying process.

EXPERIMENTAL

Colorimetric Determination of Fatty Acids:

A possible colorimetric means of determining fatty acids is to convert them to esters and then determine the esters as hydroxamic acids, as suggested by Hill (1946). He converted fatty acids after methylation to hydroxamic acids which, with ferric ions in an acid solution, give a red colour.

In addition to the method suggested by Hill, there are numerous other means of determining fatty acid esters colorimetrically (Bauer & Hirsch 1949, Hack 1955, Rapport & Alonzo 1955, and others), all founded on the technique described by Feigl (1946). We tested the methods mentioned above. In our hands, the procedure described by Rapport & Alonzo was found to be the simplest in use. It is readily reproducible and gives the highest degree of accuracy.

In the course of our investigation, a method was evolved of converting fatty acids to hydroxamic acids. Two means of doing this were tried. One was to transform fatty acids to acid chlorides which react with hydroxylamine to hydroxamic acids. The fatty acids were chlorinated with an excess of thionyl chloride in ether or benzene, after which the solvents were evaporated and hydroxylamine added. Comparative tests with equivalent amounts of tripalmitin gave a yield of 75 to 85 per cent. Reproducibility was poor. No improvement in either yield or reproducibility was obtained by adding pyridine.

The other method tested was the conversion of fatty acids to methyl esters and determination of the esters with the hydroxamic acid technique. The procedure suggested by Hill, first to transform the fatty acids dissolved in ether to acid chlorides with thionyl chloride and then to methylate the acids by adding methanol, was tested. This procedure gave a yield of approximately 85 per cent. If, on the other hand, the acids were chlorinated directly in methanol, the yield was approximately 94 per cent.

The reaction was tested with different fatty acids, and the results are compiled in Table I. Equivalent amounts of fatty acids and triglycerides were compared. The yield for fatty acids with more than 14 carbon atoms lay between 93 and

Table I.

Fatty acid	Yield per cent
Lactic acid	0
Butyric acid (4)	0
Caproic acid (6)	0
Caprylic acid (8)	0
Capric acid (10)	0
Lauric acid (12)	0
Myristic acid (14)	75
Palmitic acid (16)	94,5
Stearic acid (18)	93,0
Oleic acid (18)	95,2
Linoleic acid (18)	94,5

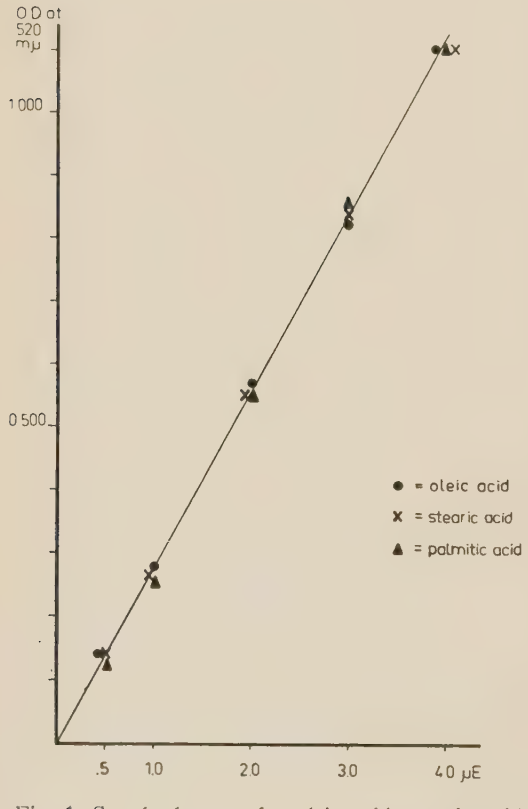

Fig. 1. Standard curve for oleic acid, stearic acid, and palmitic acid. Each point on the diagram represents the mean value of 6 duplicate tests. The curve is seen to be linear for amounts up to 4.0 μEq fatty acid.

95 per cent, no difference being noted between saturated and unsaturated acids. Recovery for myristic acid was 75 per cent, and for fatty acids with even fewer carbon atoms the yield was nil.

Different fatty acids were determined in different concentrations and a standard curve is given in Fig. 1. The method is readily reproducible and the methodic error is ± 2 per cent for amounts between 0.5 and 4.0 μEq.

Ion Exchanger Separation of Fatty Acids:

The technique of separating fatty acids from other lipids by partition between dilute alkaline ethanol and petroleum ether and reextraction of the acids to petroleum ether after acidification of the ethanol is accurate but cumbersome if many samples are to be analysed. There is another possibility — to separate the fatty acids with the ion exchanger technique, as was done by Cason, Sumrell & Mitchell (1950). The same method was also used by Borgström (1952 b, 1954) and Savary & Desnuelle (1954). In his investigation, Borgström (1952 b) found that separation of fatty acids by partition gave a better quantitative yield than ion exchanger separation. The difference is, however, slight in comparison with the advantages of the ion exchanger technique in handling a large number of samples.

The technique described by Borgström (1954) of adsorbing fatty acids in "wet" ether and eluting with a mixture of "wet" ether and acetic acid was tested. The fatty acids were found to be retained quantitatively on the ion exchanger but elution gave a poor yield. This was probably due to our use of appreciably smaller amounts of fatty acids, so that losses were more noticeable. However, by "drying" the ion exchanger with 95 per cent ethanol and equilibrating this with ethanolic ether and eluting with acid ethanolic ether, the yield was satisfactory (98 per cent). Some data from these experiments will be found in Table II.

Savary & Desnuelle used the technique of first "drying" the ion exchanger with 95 per cent alcohol. They then adsorbed the fatty acids in ether and eluted with a mixture of ethanol, ether, and hydrochloric acid. This mixture contained approximately 37 per cent (v/v) ethanol. They found the fatty acids to be eluted partially as esters. With our technique of separating fatty acids, in which

ble II. *Separation of Fatty Acids on IRA 400.*

	Adsorption	Elution	Elution time hours	Yield per cent
et" ion hanger	"Wet" ether	Ether - hydrochloric acid	2	56
		Ether — acetic acid	2	70
		Ether — acetic acid	18	86
ied" ion hanger	Ethanolic ether	Acid/ethanolic ether	18	98

ion occurs with a solution containing 5 per cent nol (v/v), we found no noteworthy esterification atty acids on passage through the ion exchanger. We also investigated whether any hydrolysis of occurred during passage through the ion exnger. Both pure triglycerides and serum extracts e passed several times through the ion exnger and the acid ethanolic ether eluate was ed for the presence of fatty acids. This techue revealed no noteworthy hydrolysis during the sage.

The reliability of the separation was tested with e substances, 2.0 mEq being set up on 15 coins. Recovery was 97.6 per cent, and the medic error ± 2.5 per cent.

Recovery Experiment:

n an experiment with fatty acids added to um, between 95 and 98 per cent were recorded.

Extraction:

Davis (1947) has stated that if extraction of y acids in petroleum ether is to be complete, plasma-water phase must be acidified down to ydrogen ion concentration of pH 6.0. In order ascertain whether or not it was necessary to lify the plasma prior to extraction, the following eriment was carried out: Plasma from 4 healthy sons and pooled plasma was extracted both ctly and after acidification to pH 5.9 with acid phosphate. The results are compiled in Tables III and IV. They show that acidification prior to extraction does not lead to any increase in yield. Boiling under reflux at different hydrogen ion concentrations was also investigated. The results of these experiments are given in Table IV. They showed acidification of plasma before extraction to be unnecessary, and also that a short boil is as effective a means of extraction as 2 hours' boiling under reflux.

Table III. *Concentration of Unesterified Fatty Acids in mEq/litre in Plasma from 4 Persons.*

Extracted at pH 7.4	Extracted at pH 5.9
0.65	0.64
0.70	0.67
0.68	0.67
0.95	1.04

Table IV. *Concentration of Unesterified Fatty Acids in mEq/litre in Pooled Plasma.*

pH	Boiled 10 secs.	Boiled under reflux 2 hours
7.4	0.67	0.67
5.9	0.70	0.70

Table V. *Methodic Error of Analysis in Duplicate.*

Unesterified fatty acids mEq/litre	Number of observations	Standard error of a single determination	per cent
0.20—0.60	36	± 0.020	10.0—3.3
0.61—1.00	72	± 0.021	3.4—2.1
1.01—2.00	29	± 0.022	2.2—1.1

Normal Series and Methodic Error:

A group of 10 healthy persons, 6 women and 4 men, aged between 20 and 35 were examined. The subjects fasted for 13 to 15 hours prior to sampling. The mean value for unesterified fatty

acids in the group was determined as 0.67 ± 0.10 mEq per litre plasma. The mean value for the unesterified fatty acids in the plasma in this small group does not differ in any great degree from that reported by other authors (Dole 1956, Gordon & Cherkes 1956, Laurell 1956, Svanborg & Svennerholm 1958). When comparing different "normal" series it should be kept in mind that the unesterified fatty acid level in plasma is highly variable.

The methodic error was calculated in over 100 analyses of duplicate samples from persons in different metabolic states. Table V shows the methodic error at different concentrations of unesterified fatty acids in plasma. The standard error of a single determination was ± 0.02 mEq per litre plasma.

DISCUSSION

The technique for extraction, purification of the extract with washing, and subsequent partition of phases and separation of phospholipids on silicic acid is the same as reported earlier from this laboratory (Carlson & Wadström 1956). In order to permit the quantitative recovery of unesterified fatty acids in the chloroform phase, however, the extract was washed with acid phosphate before partition. Experimental studies have shown that, after separation of the phases, this washing allows of quantitative recovery of unesterified fatty acids, tri-, di-, and monoglycerides, cholesterol, and phospholipids in the chloroform phase.

Different means of extracting unesterified fatty acids from plasma have been used. Davis (1947) and Grossman, Palm, Becker & Moeller (1954) used a two-phase system, in which the fatty acids were extracted from an acid aqueous phase with ether or petroleum ether. In the present investigation, the unesterified fatty acids were extracted in a single phase system with chloroform/methanol. Our studies have shown that with this procedure the plasma does not need to be acidified prior to extraction. It was also found that 2 hours' boiling under reflux gave no better a yield than a short boil. Svanborg & Svennerholm (1958) found extraction in a single phase system with chloroform/methanol to give better extraction than a two-phase system as suggested by Grossman et al. (1954). Dole (1956) also used a single phase extraction system.

Gordon, Gates & Cherkes (1957) and Svanborg & Svennerholm (1958) freeze-dried the plasma before extraction. This procedure appears to be more cumbersome than direct extraction. It is of interest to note that the unesterified fatty acid concentration in plasma in Svanborg & Svennerholm's "normal" material did not differ from that found in ours. No "normal" series comparable to that studied by us was mentioned by Gordon, Gates & Cherkes (1957).

The technique of extracting in chloroform/methanol and then purifying the extract by washing was introduced by Folch, Ascoli, Lees, Meath & Le Baron (1951), and later modified by Sperry & Brand (1955). On washing the extract with water, the fatty acids are present in both the upper and lower phase (Havel 1957). In order quantitatively to recover fatty acids in the chloroform phase after washing, it is necessary to keep the upper phase acid.

The next step, separation of the phospholipids, is necessary since a small amount (between 1 and 3 per cent) are retained on the ion exchanger and eluted from it with acid ethanolic ether. Consequently, ester linkages may interfere with the subsequent colorimetric analysis. In this connection it may be mentioned that the presence of phospholipids may also introduce a source of error in titri-

ric determination of unesterified fatty s (Garvin & Karnovsky 1956, Svanborg Svennerholm 1958).

he fatty acids were separated with the exchanger technique. The results of these eriments have shown that the fatty acids quantitatively adsorbed onto the ion ex- nger and that elution gives a satisfactory l (98 per cent). The separation permits l determination in the absence of other ls. Furthermore, these latter substances not reduced in quantity.

he final step, colorimetric determination, ows in principle the procedure suggested Hill. However, under our conditions it found better to methylate the fatty acids ctly in methanol than to use Hill's tech- ue of first chlorinating the fatty acids in r and then converting the acid chlorides methyl esters in methanol. For the hy- xamic acid reaction, we used Rapport & nzo's modification of Feigl's method.

When titrimetric methods of determining sterified fatty acids are used for long n fatty acids, interference from low- ecular fatty acids has to be prevented. procedure described in the present paper nates only long chain fatty acids and it , therefore, be said to be more specific long chain fatty acids than titrimetric niques.

SUMMARY

he present paper describes a colorimetric hod of determining the unesterified fatty s in plasma. The procedure is founded the following principles: Plasma is ex- ted in chloroform/methanol (2:1). After ation the extract is shaken with acid phos- te in a separatory funnel. The chloro- n phase is then passed through a silicic column which separates the phospho- lipids from other lipids in the extract. From this purified fat extract, the unesterified fatty acids are separated by the ion exchanger technique. The purified fatty acids are converted to methyl esters which are determined colorimetrically with the hydroxamic acid method. The error of a single determination is ± 0.02 mEq per litre. In comparison with other methods, this procedure has the advantage that the unesterified fatty acids as well as the glycerides, phospholipids, and cholesterol can be analysed in one and the same 2 ml sample of plasma. It is also highly specific for plasma unesterified fatty acids with more than 14 carbon atoms.

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QUANTITATIVE DETERMINATION OF PROTEINURIA BY CLOSS' POLYTHIONATE METHOD

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(Received for publication January 20, 1958)

In an article in a previous number of this rnal, Closs (1957) described a rapid and ple method for detection and quantitative ermination of proteinuria by means of a fosalicylic acid polythionate reagent. The sent investigation was made to control reliability of the method in quantitative ermination of relatively severe protein-as, especially in toxemia of pregnancy.

The results obtained with the polythionate thod are compared with the results ob-ned with the micro-Kjeldahl method as ference, and with Esbach's method, which the most widely used routine method in s country.

It should be pointed out that the ratio of lfosalicylic acid to urine in Closs' reagent less than a fourth of the ratio in Kings-ry & Clark's sulfosalicylic acid method, d that the protein precipitation in Closs' thod is only in small measure due to the fosalicylic acid content of the polythionate gent.

MATERIAL AND METHODS

The material used in this investigation was fresh ne from patients in a medical and a gynecologic artment of Oslo City Hospital. Protein was nonstrated in all the samples both by the boiling t and by Heller's nitric acid ring test.

Polythionate procedure.

0.6 ml urine and 6 ml of Closs' reagent (0.2 g K-polythionate and 0.2 g sulfosalicylic acid per 100 ml, pH 2) were mixed, and after 30 minutes the optical density was read against water in a Klett-Summerson photometer (Filter 66 Red 640 - 700 mμ). As the calibration curve for this apparatus was linear only up to 0.250, corresponding to a protein content of about 6 g per liter, all the samples with a higher protein content were diluted so that the readings could be made below this level.

Micro-Kjeldahl.

According to the degree of proteinuria, evaluated by Esbach's method, from 0.5 to 10 ml urine were pipetted off, and the proteins precipitated by the Folin-Wu tungstic acid precipitant (Van Slyke, 1932).

All the results presented are average values for duplicate analyses.

RESULTS AND DISCUSSION

The protein content in 38 different urines from patients with toxemia of pregnancy was determined by the polythionate, micro-Kjeldahl and Esbach methods. The results are presented in Table I.

It appears that there is good agreement between the polythionate method and micro-Kjeldahl, both for very low and very high protein values. The results obtained by Esbach's method are increasingly too low as

Table I. *Protein content in gram per liter in 38 urine samples from patients suffering from toxemia of pregnancy, determined by the Kjeldahl, polythionate and Esbach methods.*

Kjeldahl	Polythionate	Esbach
0.6	0.6	0.5
0.7	0.7	0.5
0.9	1.0	0.5
1.0	1.3	0.5
1.1	1.1	1.0
1.2	1.0	0.3
1.2	1.2	0.5
1.6	1.8	1.0
1.6	2.0	0.5
M	+0.09	—0.6
R	—0.2 – +0.4	—1.1 – +1.1
2.6	2.6	0.5
2.8	3.5	2.0
2.9	3.5	3.5
3.0	2.9	1.0
3.3	3.2	2.5
3.4	3.9	0.5
3.5	4.3	1.0
4.0	4.3	1.0
4.3	4.4	2.0
4.5	4.5	3.5
4.6	4.6	3.5
M	+0.25	—1.63
R	—0.1 – +0.8	—3.0 – +0.6
5.2	6.4	2.8
5.5	5.8	3.5
5.9	5.8	3.0
6.8	6.4	3.0
7.0	7.2	3.5
7.1	7.5	3.0
7.8	8.1	4.0
8.7	8.9	4.0
9.5	10.9	4.0
9.6	9.6	7.0
10.9	10.8	4.5
12.7	10.8	7.0
12.8	13.0	5.0
M	+0.12	—4.25
R	—1.9 – +1.2	—7.8 – —2.0
21.0	24.2	8.0
21.2	26.8	9.0
21.4	21.0	8.0
34.0	37.0	15.0
44.0	40.0	15.0
M	+1.48	—17.32
R	—4.0 – +5.6	—29.0 – —12.2

M: Mean difference from the Kjeldahl results.
R: Range of the differences from the Kjeldahl results.

Table II. *Protein content in gram per liter in 16 urine samples from patients with various types of renal affection, determined by the Kjeldahl, polythionate and Esbach methods.*

Diagnosis	Kjeldahl	Polythionate	Esbach
Nephritis chronica	0.8	0.6	0.3
»	0.9	0.5	0.3
»	1.6	1.7	1.0
»	1.9	1.9	1.0
»	2.3	2.4	1.0
»	2.3	2.8	1.0
»	2.5	2.4	1.0
»	2.8	2.9	1.0
»	4.2	4.2	2.0
»	5.1	5.0	1.8
Vit. cordis c. insuff.	0.5	0.5	0.3
»	0.8	0.5	0.5
»	0.9	0.7	0.9
Nephropathia diabeticum	1.1	1.5	0.8
»	1.4	1.9	0.8
»	1.6	2.3	1.7

the protein content increases, but it should be pointed out that undiluted urine has been used in these analyses, which is the common practice at most of the hospital laboratories in this country.

A similar comparative investigation of urine from 16 patients with various other renal affections yielded corresponding results (Table II). The greatest differences between the polythionate method and micro-Kjeldahl were found in 3 cases of nephropathia diabeticum. This may be due to an increased content in the urine of polypeptides, which are precipitated by the sulfosalicylic acid in Closs' reagent.

Increased content of bile in the urine does not influence the results with Closs' method for proteinurias of the degree investigated. Ketone bodies have no influence.

Duplicate analyses with Closs' method practically always yielded identical results.

SUMMARY AND CONCLUSIONS

)n quantitative determination of protein tent in 54 different urine samples, mostly n patients with toxemia of pregnancy, d agreement was found between Closs' ythionate method and micro-Kjeldahl, le the results obtained with Esbach's thod in undiluted urine were consider- y lower, especially in cases of severe pro- uria. As the polythionate method is ple and rapid we believe that it satisfies most of the requirements for a reliable routine method for quantitative determination of proteinuria for clinical purposes.

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Scandinav. J. Clin. & Lab. Investigation 418—426, 10, 1958.

PAPER ELECTROPHORESIS OF I^{131}-LABELLED SERUM PROTEINS

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(Received for publication July 31, 1958)

In paper electrophoresis it is necessary to concentrate the solution if the content of total protein is less than about 1 g per 100 ml. In an attempt to avoid the concentrating procedure the proteins were labelled with radioactive iodine whereby even very small amounts of protein are detectable and easily measurable by radioautography. Proteins react very readily with iodine. It has been demonstrated that both substitution and oxidation reactions take place. The formation of 3-5-diiodotyrosine has been shown (Roche & Michel, 1951). By stepwise iodination of human albumin in pH range 9—10 Hughes & Straessle (1950) found that oxidation in the sulphhydryl groups occurs first. Then the substitutions in the tyrosyl groups commence, and up to 70 per cent of the tyrosyl groups present are transformed to diiodotyrosyl before other reactions take place. By further iodination, the remaining tyrosyl groups are substituted, but simultaneously also other kinds of iodine-binding reactions occur; probably iodination of histidyl groups (Roche & Michel, 1951).

In working out this technique it was necessary to carry out investigations on the iodination of proteins and to discover how iodinated proteins behave during electrophoresis. Further comparisons were made between the results obtained by coloring the electrophoresis paper and those obtained by registering the incorporated radioactivity. These comparisons had to be carried out first with protein solutions in which the concentration of protein was higher than 1 g per 100 ml. The results are presented in this article. The application of the method to more diluted solutions will be presented in another article.

METHODS

The paper electrophoresis was carried out on horizontally stretched, free hanging papers (Sleicher & Schüll). Barbital buffer 0.10 M, pH 8.6. Voltage 2 Volts/cm. Electrophoresis time about 18 hours. The strips were stained with Amidoblack 10 B and measured with a Zeiss automatic extinction writer. The different protein fractions were separated by perpendiculars from the lowest points on the curves to the base line. The resulting areas were measured by planimeter and calculated in per cent of the total curve area. No correction was used for the varying ability of the protein fractions to adsorb the coloring matter.

Preparation of I_2^{131} solution. I^{131} was delivered by The Joint Establishment for Nuclear Research, Kjeller, Norway, as a carrier-free, non-isotonic solution of NaI^{131}. To 0.6 ml of NaI^{131} solution (100—200 micro-curie) was added 0.1 ml of 9 per cent solution of NaCl, 0.01 ml of a KI solution 1 per cent, 0.01 ml of a KIO_3 solution 1 per cent and 0.05 ml 0.1 N HCl. Five minutes after mixing 0.05 ml 0.1 N NaOH and 0.2 ml 0.1 M NH_3—NH_4Cl buffer pH 8.6 was added. The iodine solution is iodide-free and contains about 0.075 mg iodine per ml.

ation of proteins.

. Concentration of protein less than about 50 mg per 100 ml.

o 0.2 ml of a protein solution adjusted to pH s added rapidly 0.2 ml of the above mentioned e solution and the two liquids are thoroughly d. Ten seconds later 0.1 ml of a 0.1 N solution $a_2S_2O_3$ is added and well shaken. The iodina- is carried out at 0° C.

B. Concentration of protein higher than 50 mg per 100 ml.

he iodination is carried out in the same way, it is necessary to use an I_2^{131} solution with carrier-iodine than 0.075 mg per ml. Non- active iodine-iodide solution is therefore added e I_2^{131} previous to the iodination.

Concentration of protein mg per 100 ml	Concentration of carrieriodine in the I_2^{131} solution mg per ml
100	0.15
250	0.30
500	0.60
1000	1.0
5000	1.5

rmination of protein-bound iodine.

he proteins were iodinated with solutions of containing known amounts of carrier-iodine rding to the technique just described. The protein-bound iodine compounds were removed lialysis against running water for 24 hours. efficiency of the dialysis was shown by paral- nvestigation with albumin mixed with iodide aining NaI^{131}. In this investigation more than er cent of the activity added was removed ng the dialysis. The protein content and the activity of the solutions were determined be- and after the dialysis. Thereby the protein- d iodine was easily calculated.

ntitative registering of the radioactivity.

he activity of the solutions was measured by ntillation counter "Ecko". The activity on the electrophoresis strips was registered indirectly by radioautography. The radioautographs were produced by placing the paper strips against X-ray film ("Codirex Code 7" or "Ferrani") for 24 hours. The film was developed under standardized conditions: Five minutes immersion at 20° C in Kodak X-ray Developer and 20 minutes fixation in Kodak X-ray rapid acid Fixer. After drying, the radioautographs were registered in the same way as the electrophoresis strips. It was found that there is a direct proportionality between the amount of I^{131} and the registered curve area as long as the maximal optical density is less than 0.50.

Quantitative determination of tyrosine in protein.

The proteins were hydrolysed with 6 N HCl in closed glass tubes at 120° for one hour. The HCl was eliminated by distillation in vacuum. The content of tyrosine in the hydrolysate was determined according to Gale (1946) by means of a strain of *Streptococcus faecalis* (National Collection of Type Cultures, England, No. 6782) which contains a specific L-tyrosinedecarboxylase.

RESULTS

Investigations concerning the reaction between iodine and proteins. The possible reversibility of the reaction.

When a small amount of iodine is added to a solution of protein the iodine disappears as elementary iodine. The reaction:

$$\text{Iodine} + \text{Protein} \rightarrow \text{Iodine-protein compounds}$$

must therefore either be irreversible or the equilibrium must be driven to the right. Elementary iodine does not move during an electrophoresis (easily shown by means of I_2^{131}) while iodinated proteins do so. If the above-mentioned reaction is reversible, even when the equilibrium is driven extremely to the right, elementary iodine will be removed from iodinated protein during an electro-

phoresis, resulting in a loss of the I^{131} labelling. In order to decide whether the reaction is reversible or not trials were carried out to remove elementary iodine from iodinated albumin by means of sublimation technique. A solution of albumin was iodinated with I_2^{131}. The content of carrier-iodine was slightly less than the maximal amount of iodine with which the albumin could react. In this investigation no $Na_2S_2O_3$ was added. This solution of iodinated albumin was placed in the upper compartment of a diffusion chamber (Widmark flask, used for alcohol determination). Solid NaOH was introduced to the bottom of the flask. In simultaneous control experiments a solution of I_2^{131} was used instead of iodinated albumin. After 24 hours the water had diffused over to the sodium hydroxide. Estimation showed that no radioactivity had left the iodinated albumin while in the control experiment all activity had diffused over to the sodium hydroxide. The conclusion to be drawn from these investigations is that the reaction between albumin and iodine must be of irreversible nature.

The influence of hydrogen-ion-concentration on iodination of protein.

In order to estimate the pH value which is most favorable for the iodination of proteins, solutions of human albumin (presented by A/B KABI, Sweden) were iodinated at different pH values and the protein-bound iodine determined according to the technique previously described. The results (Table I) show that iodination at pH values of about 9 gives the greatest incorporation of the added iodine. This is in accordance with earlier investigations (Herriot 1947, Roche & Michel 1951) which have shown that iodination in weak alkaline milieu primarily causes substitution reactions, while iodination in acid milieu mostly causes oxidations.

Comparison between the ability of different serum proteins to incorporate iodine.

The method presented in this paper is based on the assumption that the relative concentration of the different protein fractions may be determined by registering the amount of iodine incorporated in each fraction. In order to examine the ability of different proteins to incorporate iodine, different solutions of isolated serum proteins (presented by A/B KABI, Sweden) of a concentration of 20 mg per ml, were labelled with radioactive iodine at pH 8.6. The protein-bound iodine was estimated according to the technique described above. The results, shown in Table II, demonstrate that all serum proteins investigated have nearly the same uptake of iodine per gram protein. It must therefore be assumed that one will get an approximately correct expression for the relationship between the different protein fractions by recording the relationship between the amounts of iodine incorporated in each protein fraction.

Table I. *Incorporation of iodine in human albumin at different pH values.*

pH	4.00	5.71	6.42	7.25	7.65	8.55	9.22	10.45
Mg iodine incorporated per gram of albumin	2.1	19.4	19.8	25.0	29.8	30.8	30.8	27.5

ble II. *Comparison between the ability different human serum proteins to incor- ate iodine and their content of tyrosine.*

	Albumin	α-lipo-protein	β-lipo-protein	γ-globulin
iodine rporated	32.0	26.3	34.0	31.4
gram of ein	33.0	26.8	36.0	31.0
tent of sine in gram of protein n in grams	7.68 7.95	4.90 4.80	6.82 6.92	10.42 10.42

Even if the tyrosyl groups are considered many investigators to be the most impor- t iodine acceptor in proteins, other forms incorporation of iodine have to be borne mind (Roche & Michel 1951). It was refore of interest to study the relationship ween the content of tyrosyl groups of pro- ns and their ability to incorporate iodine. ble II shows the percentage of tyrosine the examined proteins. A poor correlation s found between the content of tyrosine the amount of iodine incorporated. This estigation strengthens the assumption t also other groups than the tyrosyl groups ction as iodine acceptors.

e possible influence of the iodination of proteins on their behaviour ring electrophoresis.

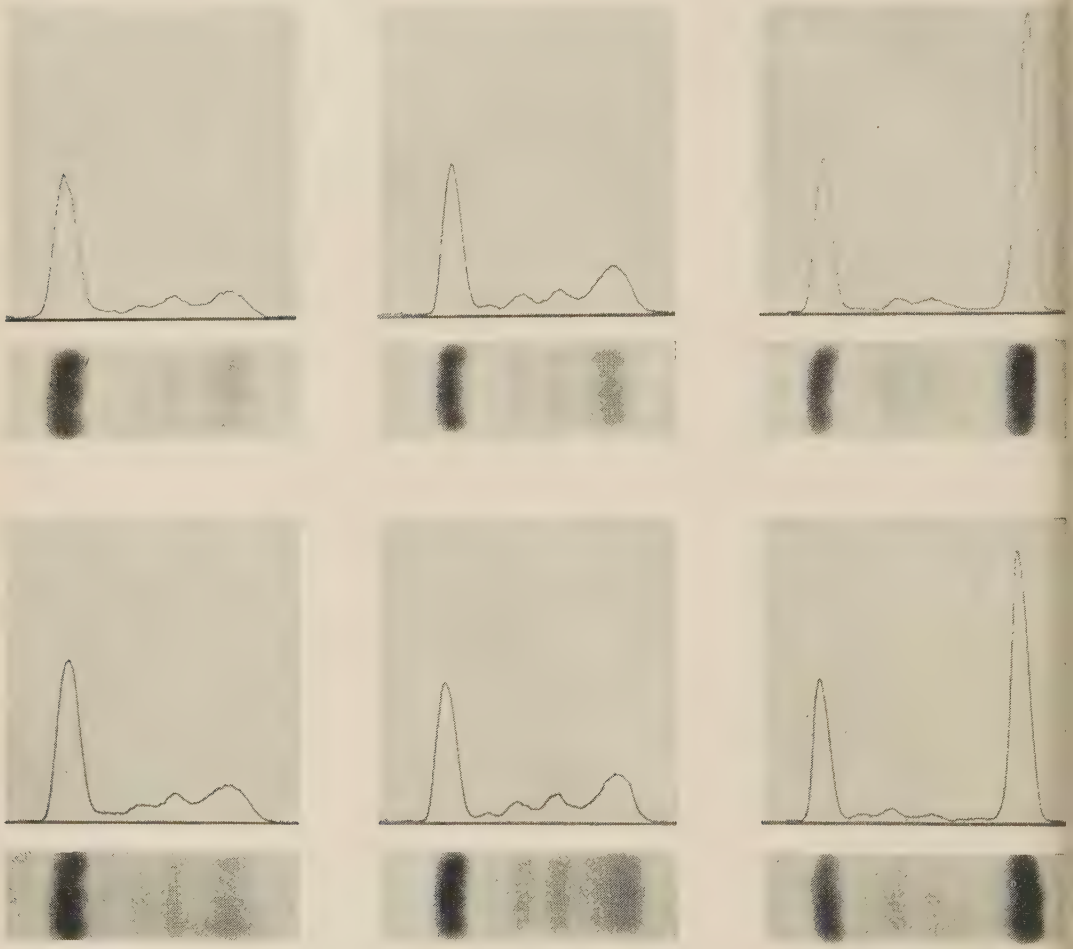
The chemical structure of proteins is nged by iodination, and it is possible, refore, that iodinated proteins behave dif- ently during electrophoresis to the cor- ponding native ones. In order to investi- e this matter, paper electrophoresis was ried out with native as well as iodinated tein solutions and sera. Twenty different sera, normal as well as pathological, were examined. It was found that the iodination had no influence whatsoever. The results obtained from the native sera correspond perfectly with those obtained from the iodinated sera. The difference found was within the limits of variation for repeated analyses of the same serum determined by Sommerfeldt (1952). Fig. 1 shows three of the examined sera together with the corresponding results. These investigations show that labelling of proteins with I^{131} does not influence their behaviour during electrophoresis.

Formation of iodide in the reaction between iodine and protein.

A solution of albumin was iodinated with a very small quantity of iodide-free iodine labelled with I_2^{131}. The amount of iodine added was so small that nothing remained as elementary iodine. This solution of iodinated albumin was subjected to paper electrophoresis of short duration (one hour, 2 Volts/cm). Fig. 2 a shows the radioautograph of the strip before treatment with acetic acid-methanol. Fig. 2 b shows the radioautograph of the strip after the treatment with acetic acid-methanol. Fig. 2 c gives the reproduction of the strip after coloring with Amidoblack 10 B. These investigations show that the iodine which has reacted with albumin separates into two fractions during electrophoresis. One fraction follows the albumin and remains incorporated in it during treatment with acetic acid-methanol. The other fraction moves more rapidly than the albumin and is dissolved in the fixing bath with acetic acid and methanol. This fraction probably consists of iodide ions. The correctness of this assumption is shown in the following experi-

Fig. 1. *Paper electrophoresis of untreated human sera and of the same sera saturated with iodine.*

Upper line: untreated sera. — Lower line: iodinated sera.



Serum No. 1 2 3

	Native sera					Iodinated sera				
Serum	Alb.	α_1	α_2	β	γ	Alb.	α_1	α_2	β	γ
1	57.5	4.0	10.8	12.3	15.4	57.2	5.7	10.8	12.1	14.2
2	46.7	5.1	9.6	13.8	24.8	46.5	5.2	10.0	12.4	25.9
3	33.3	3.8	5.7	5.4	51.8	34.2	3.2	6.0	5.7	50.9

Fig. 2. *Paper electrophoresis of human albumin iodinated with I^{131}. One hour duration. Voltage: 2 Volt/cm.*

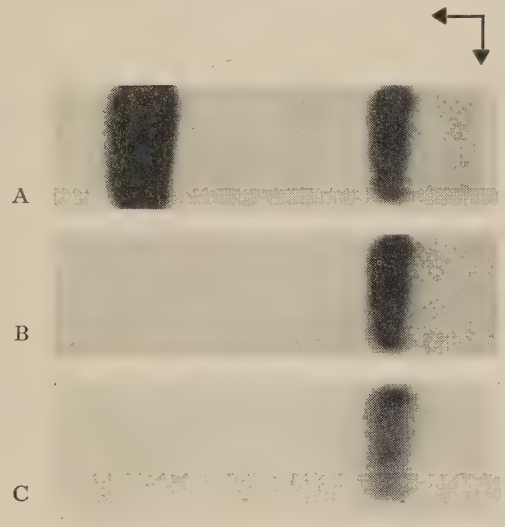

A
B
C
Radio autography before washing the paper with methanol — acetic acid
Radio autography after washing the paper with methanol — acetic acid
Paper colored with Amidoblack 10 B

Fig. 3. *Paper electrophoresis of I^{131}-labelled human albumin, I^{131}-labelled human gamma globulin and a I^{131}-labelled mixture of these two proteins.*

Upper line: Strips colored with Amidoblack.

Lower line: Radio autographs prepared from the same strips.

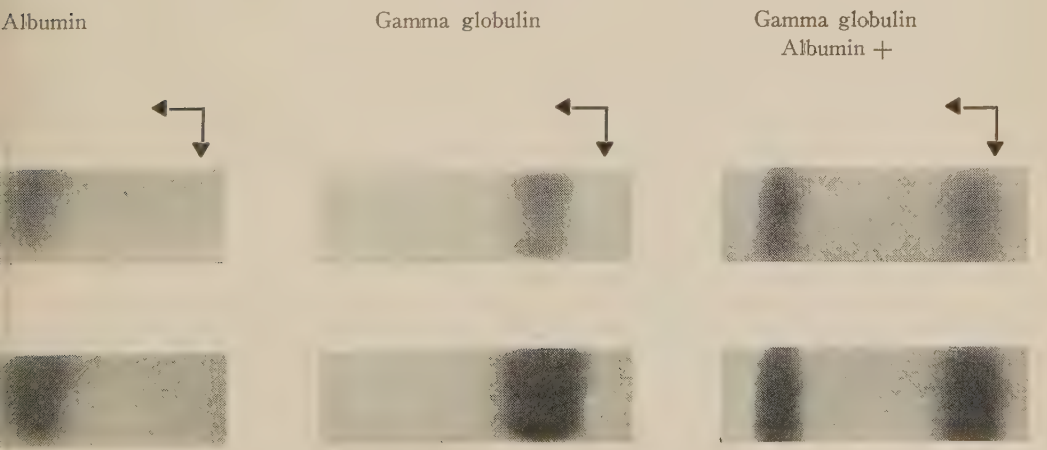


Table III. *Paper electrophoretic analyses of a mixture of human albumin and human γ-globulin registered both by radioautography and staining the strips. (The mixture: Albumin 1.94 gram per 100 ml, γ-globulin 2.16 gram per 100 ml.)*

	Stained strips		Radioautographs	
	Albumin	γ-globulin	Albumin	γ-globulin
Average of 10 parallel analyses	56.1 %	43.9 %	50.3 %	49.7 %
Range	53.2 — 58.9	41.1 — 46.8	49.0 — 51.2	48.8 — 51.0
The real values	47.3	52.7	47.3	52.7
Difference between found and real values	+8.8 %	—8.8 %	+3.0 %	—3.0 %

ment. The solution of iodinated albumin was subjected to electrophoresis at pH 5.0 (acetate buffer). Straight across the paper, near the anode basin, a stroke of a solution of $AgNO_3$ was drawn. During the electrophoresis the silver ions move in the direction of the cathode, *viz.* in the opposite direction of the I^{131}-containing compounds. It was shown that the "rapidly moving fraction" completely stopped when it met the silver ions. The resulting combination remained quantitatively fixed to the paper after treatment first with HNO_3 and then with NH_3. In this way it was proved that the "rapidly moving fraction" consists of iodide ions. The results of this investigation are in full agreement with the chemical equations for substitution and oxidation of proteins with iodine, showing that iodide is produced from iodine during its reaction with proteins.

Table IV. *Paper electrophoretic analyses of a mixture of human albumin, human α-lipoprotein and human γ-globulin registered both by radioautography and staining the strips. The concentration of each of the three proteins in the mixture was 2 gram per 100 ml. As the α-lipoprotein did not completely separate from the γ-globulin, both fractions are combined.*

Stained strips			Radioautographs	
	Albumin	α-lipoprotein + γ-globulin	Albumin	α-lipoprotein + γ-globulin
Average of 10 parallel analyses	38.5 %	61.5 %	31.8 %	68.2 %
Range	36.3 — 41.4	58.6 — 63.7	30.4 — 33.6	66.4 — 69.6
The real values	33.3	66.7	33.3	66.7
Difference between found and real values	+5.2	—5.2	—1.5	+1.5

Experiments with isolated proteins.

Isolated proteins (presented by A/B KABI, Sweden) were iodinated as described previously. After electrophoresis the strips were treated with acetic acid-methanol in order to remove all non-protein-bound iodine compounds. Fig. 3 shows the results obtained by electrophoresis of albumin, γ-globulin and a mixture of these two proteins. Table III gives the quantitative results of investigations carried out in a known mixture of albumin and γ-globulin. The amounts of protein used for preparing the mixture were determined by the method of Kjeldahl. Table IV gives the corresponding results in a known

Fig. 4. *Paper electrophoresis of untreated human sera and the same sera iodinated with I^{131}.*

er line: Untreated sera. Papers colored with Amidoblack.
er line: Iodinated sera. Radioautography of the papers.

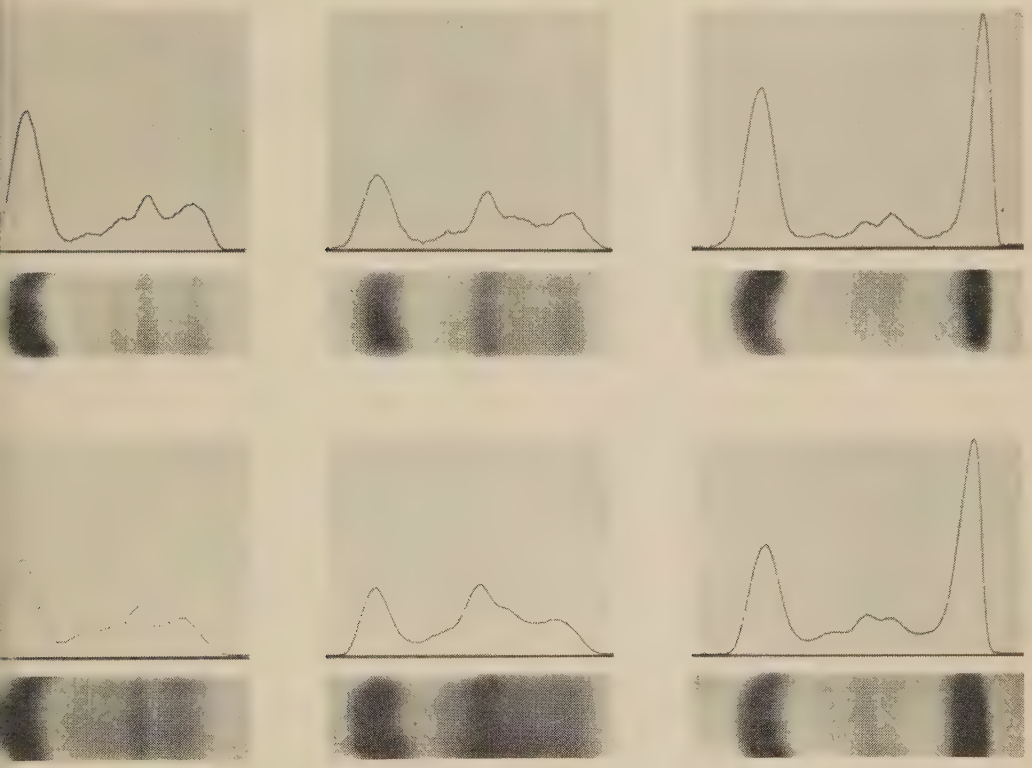


Table V. *Paper electrophoretic analyses of human sera. Carried out both with non-iodinated sera (recorded by coloring the strips) and with the same sera after labelling with I^{131} (recorded by radioautography). Each figure in the table is an average value obtained from 10 sera. All analyses were performed in duplicate.*

	Albumin	α_1-globulin	β-globulin	α_2-globulin	γ-globulin
Non-iodinated sera	50.6	4.6	9.2	10.6	25.0 per cent
I^{131}-labelled sera	43.5	6.6	12.5	11.7	25.7 per cent
Difference	+ 7.1	—2.0	—3.3	—1.1	—0.7

mixture of albumin, α-lipoprotein and γ-globulin. It seems that registering the incorporated I^{131} gives a more correct result than registering the amount of Amidoblack absorbed by the proteins.

Experiments with human sera.

Ten different human sera were subjected to paper electrophoresis both in native and in I^{131} labelled condition. Fig. 4 gives the results of three examined sera. Table V gives a summary of the results of these quantitative investigations. These investigations show that there is no fundamental difference between the results obtained by radio-autography and those obtained by coloring the strips. Quantitatively, a small difference is shown. The albumin fraction is found to be somewhat lower when measured by means of radioautographs than by means of colored strips.

SUMMARY

The present investigations were carried out for the purpose of working out a method for direct paper electrophoresis of protein in solutions of very low concentration. It was planned to base such a method upon labelling the proteins with I^{131} and registering the separated fractions of protein by means of the incorporated radioactivity. In this paper, preliminary experiments with protein solutions of higher concentration are reported. Quantitative determination of I^{131} by means of radioautography was investigated. Direct proportionality between the amount of I^{131} and the intensity of the blackening on the radioautograph was obtained as long as the measured optical density did not exceed 0.50.

Studies were carried out on the iodination of proteins. It was shown that the reaction between protein and iodine is irreversible. It was found that the most favorable pH value when iodinating proteins is about 9. By determining the amount of iodine incorporated in different isolated serum protein fractions under standard conditions, nearly the same uptake of iodine per gram protein was found.

Furthermore it was demonstrated that iodination does not produce any perceptible influence on the electrophoretic pattern. Quantitative measurement of the different protein fractions, using radioautography and the ordinary staining technique, gives somewhat lower albumin values with the former method as compared with the latter. Electrophoresis of mixtures of isolated serum proteins of known concentrations shows that the results with radioautography are more correct than those found by registering the stained strips.

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A NOTE ON THE COPPER SULFATE METHOD OF SERUM PROTEIN DETERMINATION

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(Received for publication May 2, 1958)

n van Slyke's method for serum protein ermination the specific gravity of the um is estimated by observing the behav- of a falling serum drop in a series of per sulfate solutions with known specific vities. As to the correlation between the tein concentration and the specific gravity the serum a great number of different mulae have been proposed, all of the same eral type: $P = a\ (G - b)$, where P ans the protein content in gm per 100 ml serum, a and b are various constants, ereas G stands for the specific gravity of serum measured as D_t^t, *i. e.* the gravity ° C with the gravity of water at the same perature as unit. The specific gravity is stly expressed as $D\frac{25}{25}$.

n Sweden the preparation of the stock per sulfate solution is carried out accord- to the Swedish Pharmacopoeia, where the cific gravity of a solution is defined as gravity of the solution at 20° C compared h the gravity of water at 4° C. The stock per sulfate solution is thus made with the cific gravity of 1.1000 as D_4^{20} instead of 000 as D_{25}^{25} (corresponding to 1.0983 $D\,{}^{20}_{4}$).

n order to estimate the possible errors in serum protein determinations when using per sulfate solutions with inaccurate spe- c gravities the following three experi- nts were carried out.

. From a stock solution of copper sulfate with specific gravity of 1.1000 as $D\,{}^{25}_{25}$ a series of ions was made according to the approximate rule: 1 ml less than the second and third decimal in the desired specific gravity is diluted to 100 ml with distilled water. This procedure is recommended by Plimmer (1944). The resulting specific gravities were measured pycnometrically as $D\,{}^{25}_{25}$. The relationship between the supposed and the measured gravities is given by line A in the figure.

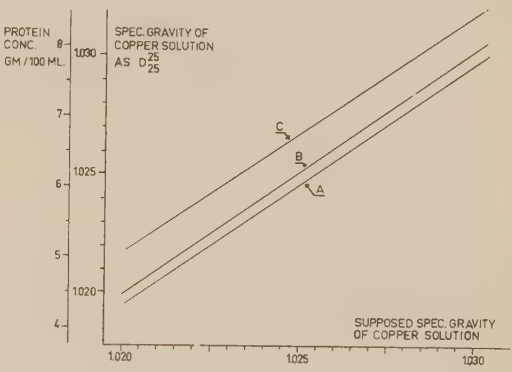

Fig. 1. Relationships between the supposed specific gravities of the copper sulfate solutions after different dilution procedures and the protein concentration calculated from the pycnometrically measured specific gravities.

Line A: Dilutions according to approximate rule from stock solution with $D\,{}^{25}_{25} = 1.1000$.

Line B: Dilutions according to approximate rule from stock solution with $D\,{}^{20}_{4} = 1.1000$.

Line C: Dilutions from stock solution with $D\,{}^{20}_{4} = 1.1000$ exactly to desired specific gravites as $D\,{}^{20}_{4}$.

2. The same series of dilutions was then made starting from a stock solution with the specific gravity of 1.1000 as $D\,{}^{20}_{4}$. This procedure has hitherto erroneously been used in some laboratories. The resulting specific gravities measured pycnometrically as $D\,{}^{25}_{25}$ were plotted against the supposed gravities (line B in the figure).

3. In the last experiment the dilutions were made from the stock solution with the specific gravity of 1.1000 as D^{20}_{4} exactly to the desired specific gravities measured as D^{20}_{4} and not according to the approximate rule. The resulting specific gravities were then measured as D^{25}_{25}. The relationship between the specific gravities as D^{20}_{4} and D^{25}_{25} is seen as line C in the figure.

It was then assumed that the three series of copper sulfate solutions were to be used for the determination of the serum protein concentration according to van Slyke's method. The protein concentration corresponding to the various copper sulfate solutions was calculated from the formula $P = 343\ (G - 1.0070)$ suggested by Moore & van Slyke (1930). G is the specific gravity as D^{25}_{25} of the copper sulfate solution in balance with the serum drop. A scale for the calculated protein concentration is shown in the figure.

The following conclusions can be drawn from these experiments. If the stock solution prepared with the specific gravity of 1.1000 as D^{20}_{4} is diluted according to the approximate rule without control of the attained specific gravities (line B), the calculated protein concentrations are about 0.2 gm per 100 ml lower than when the dilutions are made with the stock solution with a specific gravity of 1.1000 as D^{25}_{25} (line A). If, on the other hand, the dilutions of the stock solution with $D^{20}_{4} = 1.1000$ are made to the exact gravities as D^{20}_{4} (line C), the protein values will be about 0.6 gm per 100 ml lower than when using the stock solution with $D^{25}_{25} = 1.1000$.

It is thus of great importance that the copper sulfate solutions used in the protein determinations be prepared strictly according to the original recommendation, especially if a formula is to be used in the calculation of the protein content.

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Scandinav. J. Clin. & Lab. Investigation 429—431, 10, 1958.

NEW DESIGN OF VOLUMETRIC RESPIROMETER

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(Received for publication March 19, 1958)

There are certain merits inherent in volumetric microrespirometry which conventional volumetric systems lack, namely, that calibration is independent of the vessel volume, the system can easily be compensated for all temperature and pressure variations. The principle has not been generally used, for one reason, because no design hitherto proposed has been simple to make. This objection has been largely removed in the present construction, which retains the same versatility of operation as the original design (Scholander, Claff, Andrews, & Wallach, 1952).

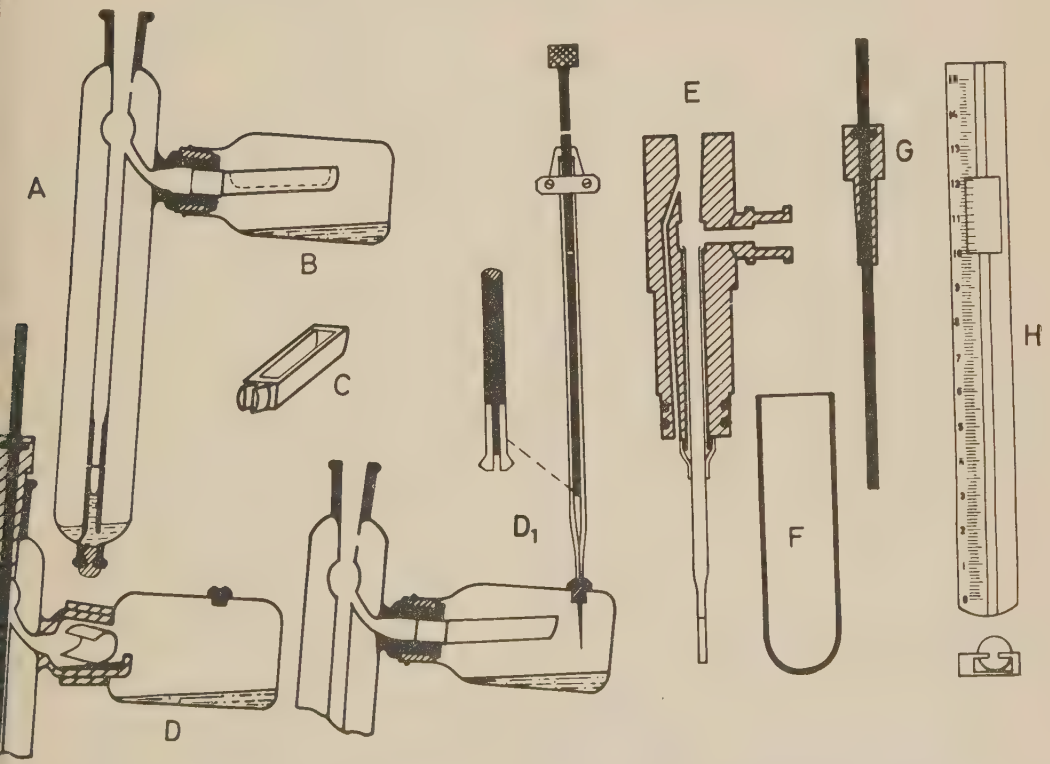

Fig. 1. A—B: Glass respirometer, without rod. C: Detachable tray for CO_2 absorption. D: Reaction vial with CO_2 absorber in hollow stopper. D_1: Volume compensated injection. (Detail shows polyethylene tip of injection microsyringe.) E: Longitudinal section of plastic respirometer with chamber tube inserted and detached compensating chamber: F. G: Rod plug with "O" ring and H: Vernier scale reader with sliding indicator to engage top of rod, while rule rests on plug.

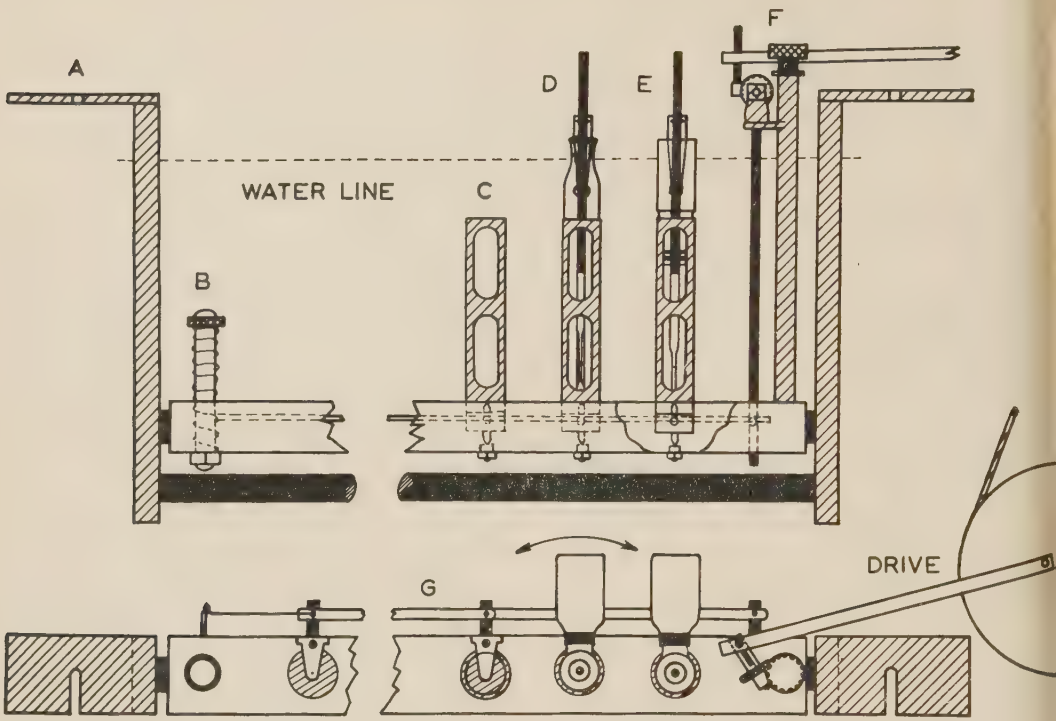

Fig. 2. Shaking rack for respirometers. A: Frame to be clamped on water bath. B: Spring adjustment for shaking mechanism. G: Tube holder for respirometer. D: Glass respirometer in holder. E: Plastic respirometer in holder. F: Drive and tilt mechanism. G: Link connection between each unit.

The respirometers may be made of either glass or plastic. The shaking arrangement is concentric and does not interfere with setting and reading the manometer rod. The rates of temperature equilibration and oxygen diffusion are the same as in the previous edition, and the rate of CO_2 absorption is about doubled.

The respirometer (Fig. 1, A or E) consists of a rod chamber which is connected with the reaction chamber (B) by a side bore. The rod chamber continues down through the middle of the compensating vessel (A and F), and constricts in the lower part to a capillary with an index mark. The opening of the capillary dips into the manometer fluid covering the bottom of the compensating chamber. The compensating chamber of the plastic model (F) is cylindrical and fits into the rod chamber by means of two "O" rings, or if the instrument is all glass the compensating chamber is built around the rod chamber (A). The rod slips air tight through the plug by means of an "O" ring (G), and when seated it closes the vent hole to the compensating chamber. The rod plug has a bevel which can be turned so that the compensating chamber and the rod chamber interconnect. The reaction chamber, which may have any suitable shape, connects with the rod chamber by means of a Neoprene ring (B), or ground glass joint (D). CO_2 is absorbed by KOH on a filter paper placed in an elongated plastic cup, which is detachable (C), or it can be built into the ground glass connector (D).

The respirometers fit into a rack (Fig. 2) with tubular holders which pivot concentric-

on points. The holders are connected by
s, and can be agitated with variable am-
ude at 3 different constant speeds. The
k can be given a suitable tilt by a lock
w at F.

The operation of the respirometer is the
e as described previously, and all opera-
s, such as compensated injection, removal
fluids (Fig. 1, D_1), intermittent absorp-
for RQ determinations, flushing with
gen, *etc.*, are performed exactly as in the
lier design. The new model, provided with
arrower rod, can also be used for direct
erminations of plasma carbon dioxide, ac-
ding to the method of Kinoshita, Bunker
Scholander (1952).

ACKNOWLEDGEMENTS

We wish to acknowledge our indebtedness to Mr. J. D. Graham, Physiology Department of the School of Medicine, University of Pennsylvania, and Mr. J. Andresen at the Central Institute for Industrial Research, for executing the glass parts of the respirometers.

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Scandinav. J. Clin. & Lab. Investigation 432—434, 10, 1958.

DETERMINATION OF N-(5-NITRO-2-FURFURYLIDENE)-1-AMINOHYDANTOIN IN URINE

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(Received for publication May 24, 1958)

During the last few years some derivatives of nitrofuran, *e. g.* nitrofurantoin /N-(5-nitro-2-furfurylidene)-1-aminohydantoin/, Furadantin (R), have been widely used in the treatment of urinary infections. A method for the determination of this substance in urine has been described by Bender, Nohle & Paul (1956). According to these authors the estimation is performed by measuring the optical density at 370 mμ; or after having formed the phenylhydrazone of nitrofurfuraldehyde at 420 mμ. However, in order to avoid interfering substances the nitrofurantoin has to be isolated by a chromatographic procedure. In the method described below, this has been substituted by an extraction procedure, based upon the observation that nitrofurantoin, with a quarternary ammonium salt, *e. g.* benzethonium chloride, gives a colored compound which can be extracted with chloroform from a water solution. The determination is then performed by measuring the extinction of the chloroform solution at 400 mμ.

METHOD

Reagents

Buffer pH 6.0. Mix 877 ml of 0.1 M KH_2PO_4-solution with 123 ml of 0.05 M $Na_2B_4O_7$-solution.

Benzethonium chloride, 1 per cent solution in chloroform. Dissolve 10 g benzethonium chloride in 1000 ml of chloroform p. a.

Procedure

Transfer 5.00 ml of urine to a 100 ml separatory funnel containing 50 ml of buffer solution pH 6.0. Extract the water solution three times with 20 ml of 1 per cent benzethonium chloride solution and then with 10 ml portions until the chloroform is colorless. Vigorous shaking should be avoided, because emulsions might be formed which will greatly increase the time of separation. Filter the combined chloroform extracts through two filter papers, Munktell 00, moistened with benzethonium chloride solution and dilute to 100 ml with the same solution. Transfer 3.00 ml to a 1 cm cuvette and measure the extinction at 400 mμ using 3.00 ml of the benzethonium chloride solution as a blank. Add 0.100 ml of glacial acetic acid to the sample as well as to the blank and read the extinction again. Correct the last reading for dilution by multiplying by 1.033. The difference between the extinctions before and after the addition of acetic acid is a function of the nitrofurantoin concentration.

A standard curve or factor is obtained from determinations performed on urine with known concentrations of nitrofurantoin. If the amount present is greater than 20 mg per 100 ml of urine the quantitative extraction is more difficult, therefore less urine should be taken. When analyzing urines with very low concentrations the amount of benzethonium chloride solution can be lowered, or a long cuvette, *e. g.* 50 mm, may be used.

RESULTS

The following table gives some figures demonstrating the recovery of nitrofurantoin from different urines (1 cm cuvette). The determination was made on 5 ml of urine and the final volume was 100 ml.

Added mg 100 ml	Found mg 100 ml
0.8	1.0
2.0	2.0
4.0	3.9
4.4	4.4
10.5	10.6
15.8	16.0
20.3	20.7

DISCUSSION

enzethonium chloride is a synthetic deter- t of the cation type, and it is mainly used l germicide. For the determination of sur- active ionic substances Mukerjee (1956) l ionic dyes which combine to a compound ch, in contrast to the separate compo- ts, can be transferred from water to an anic solvent. Thus quarternary ammo- m salts and long chained amines can be rmined in water solutions through addi- of bromphenol blue at a suitable pH, raction with chloroform and measuring optical density. Darawy & Tompsett 56) relate a similar procedure for the rmination of alkaloids in biological ma- al. This principle seems to be generally d to get ionic substances occurring in bio- cal material available for analysis. It is, vever, a great advantage if the substance e determined is the colored part of the med compound. This principle has been ized in the present paper in a method ch is mainly intended for control of nitro- antoin therapy. The accuracy and the sitivity are sufficient for this purpose. der, Nohle & Paul (1956) report that r a dose of 100 mg to normal patients a ximum urinary concentration varying be- en 4 and 48 mg per 100 ml was found. have given doses of 50 mg and after 3 rs the urinary concentration was found be between 4 and 28 mg per 100 ml in ferent patients.

he combination between nitrofurantoin benzethonium chloride is dependent upon . When the pH of the water phase was ied between 5.0 and 11.4 the absorption ximum of the compound in chloroform s found at 400 mμ (Fig. 1, curve 1). If pH of the water phase is lowered to 4.2 absorption curve in chloroform is sup- ssed and the maximum is shifted towards er wavelengths (Fig. 2). This indicates at this pH a mixture of nitrofurantoin the nitrofurantoin-benzethonium chlo- compound is extracted with the chloro- n. If glacial acetic acid is added to the

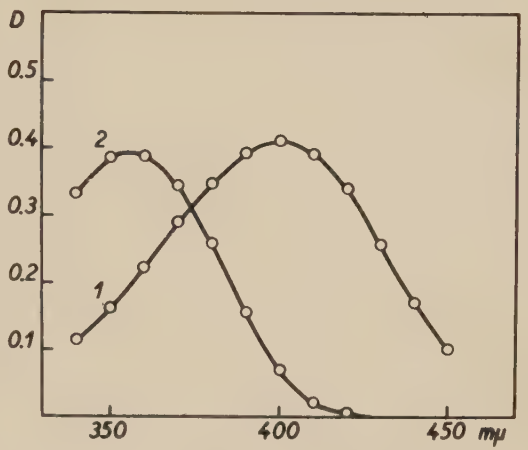

Fig. 1. Absorption curves of the nitrofurantoin-benzethonium compound in chloroform, extracted from a water solution of pH 6.0. (1) before, and (2) after addition of glacial acetic acid. (2) identical with nitrofurantoin in chloroform.

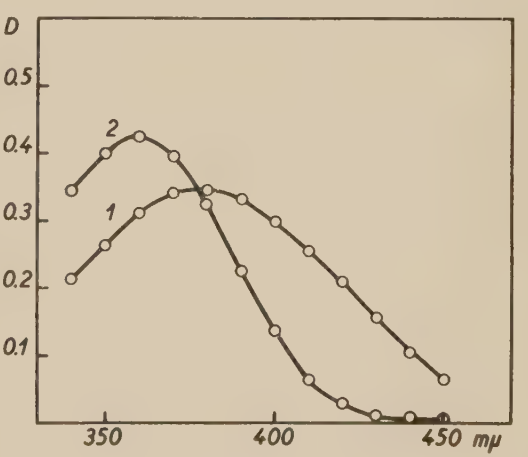

Fig. 2. Absorption curve of the nitrofurantoin-benzethonium compound extracted from a water solution of pH 4.2. (1) before and (2) after addition of glacial acetic acid.

chloroform extract an absorption maximum at 355 mμ is obtained, which is identical with the maximum obtained from pure nitrofurantoin in chloroform (Fig. 1, curve 2). The absorption at 400 mμ is then very low. Provided that the urinary blank is not altered

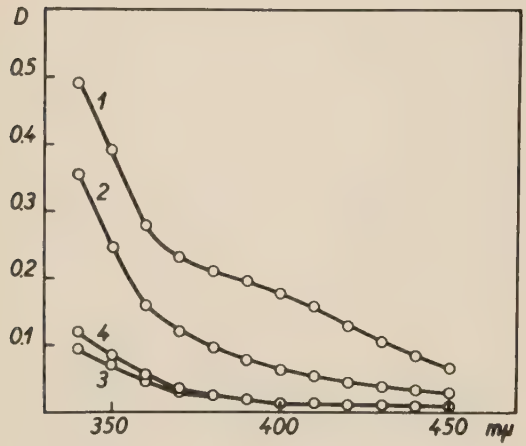

Fig. 3. Urinary blanks. (1) and (2): urine, pH 11.4. (3) and (4): urine, pH 6.0. (1) and (3) before, and (2) and (4) after addition of glacial acetic acid.

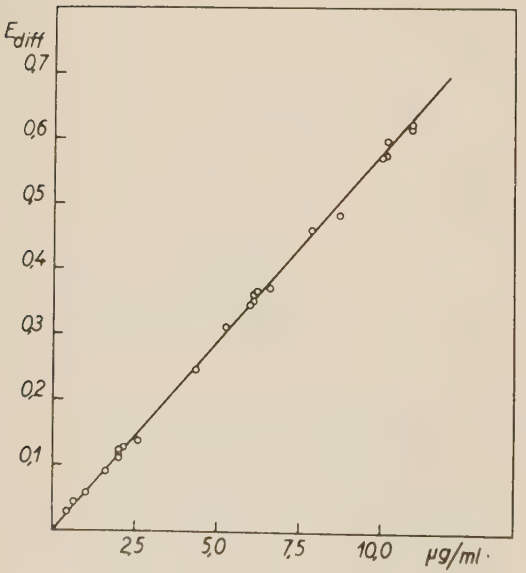

Fig. 4. Standard curve for determination of nitrofurantoin in urine. The change of extinction at 400 mμ of the chloroform extract on acidification as a function of nitrofurantoin concentration.

by the addition of glacial acetic acid this blank can be eliminated by measuring the difference between the extinctions at 400 mμ before and after addition of acetic acid. That this assumption is valid can be seen from Fig. 3 which shows the magnitude of this change in optical density at 400 mμ of chloroform extract of urines of different pH.

It was found that a direct extraction of nitrofurantoin from urine is difficult. Probably the salts occurring in the urine interfere. A dilution of the urine with buffer solution, which in this method is made prior to the extraction eliminates this drawback.

The compound of nitrofurantoin and benzethonium chloride is sensitive to light. This is shown by the following values obtained on a chloroform solution of the colored compound containing about 10 mg per 100 ml calculated as nitrofurantoin.

Time in minutes	E_{diff} in light	E_{diff} in darkness
0	0.483	0.483
10	0.454	-
30	0.363	0.493
60	0.310	0.497
180	0.244	0.488

It is evident that the compound is stable if protected against light.

SUMMARY

A method for determination of nitrofurantoin in urine is described. The method is based upon the formation of a yellow compound of nitrofurantoin and benzethonium chloride that is soluble in chloroform, and the photometric determination of the color.

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METHOD FOR DETERMINING ABSOLUTE RETICULOCYTE COUNT

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(Received for publication July 12, 1958.)

No simple acceptable method using a nting chamber has hitherto been available assessing the number of reticulocytes in olute values. The procedure described by edlander and Wiedemer (1929) is less isfactory than conventional methods using od smears, the reticulocytes not staining tinctly enough to be recognised with cer- nty under the dry high power lens.

It is obvious that a simple reliable method determining the absolute reticulocyte nt has many advantages. Knowledge of number of reticulocytes per unit of vo- ne of blood is a more adequate measure the production of red blood cells than ply the percentage of erythrocytes con- ning reticulum. Moreover, red cell pro- ction can only be assessed if the red cell nt is also determined, which is not always ne in routine clinical work.

METHOD

In the method described below capillary blood tained in a capillary pipette and afterwards fixed a diluent. The cells are stained with new methy- e blue introduced as a reticulocyte stain by cher in 1949. It is better than other vital stains this purpose. It differs from metylene blue in our index (927 as against 922) and has also a erent absorption maximum. The diluting fluid, ch also serves as a fixing agent, consists of po- ium thiocyanate in dilute sulphuric acid. The tion process is attended by an escape of haemo- in from the red cells, which then show up more rly than otherwise. The reticulum is well out- d.

Reagents

Staining fluid: Sequestrene NA_2 (Geigy) 1 Gm
Sodium chloride ..... 0.1 »
New methylene blue*).. 0.1 »
Distilled water ..... 100.0 »

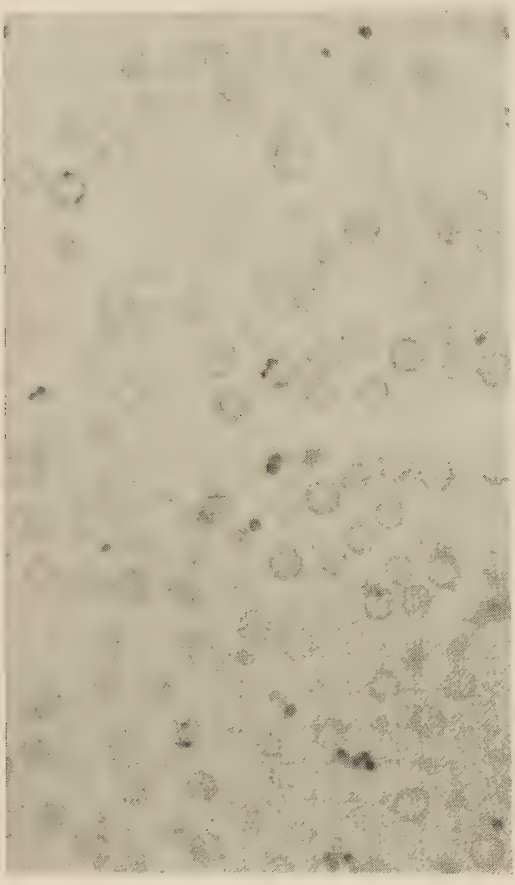
Sequestrene is neutral di-sodium salt of ethylene-diaminetetraacetic acid. The di-sodium salt «Versene» cannot be used.

Fixation fluid: 0,30 % KSCN in 0.05 normal H_2SO_4.

Procedure.

1. Aspirate into an Ellermann pipette 10 cu.mm. of staining fluid 10 cu.mm. of blood from a fresh finger prick, and finally another 5 cu.mm. of the staining fluid.
2. Suck the mixture to and fro in the pipette to ensure intermixture between staining fluid and blood.
3. Set pipette aside for 10 min. or more.
4. Blow mixture into an Ellermann tube containing 475 cu.mm. of fixation fluid.
5. Shake or rock the cell suspension for a few minutes.
6. Transfer from the suspension to a Bürker counting chamber and allow 10 minutes for cells to settle, preferably in moist chamber.
7. Count the reticulocytes in 16 C-D squares under the dry high power lens.
8. Multiplication of the number thus found by 2500 gives the absolute number of reticulocytes per cu.mm.

*) New methylene blue is manufactured by National Aniline Division, Allied Chemical & Dye Corp., New York and was supplied by A/B Grave, Grave, Stockholm.



Normal range

On examination of specimens from 104 apparently healthy adults with normal blood values (40 females and 64 males) the following values were found.

Mean: 30169 ± 1061 reticulocytes per cu.mm.

S.D.: 10817 ± 750.

This gives a normal range of 10000 — 50000 reticulocytes per cu.mm.

Comments

The suspension of red cells in the diluting fluid appears to keep indefinitely. It is not adversely affected by freezing and subsequent thawing. The reticulocytes show up clearly and are readily identified under the dry high power lens. The value of the method for counting the platelets, which are also well defined, is receiving attention.

DISCUSSION

The discovery of the fixing properties of thiocyanate in sulphuric acid was fortuitous. As mentioned, fixation is accompained by an escape of haemoglobin from the cell. This might be due to some change in the permeability of the erythrocyte membrane. The decrease in haemoglobin content enhances the definition of the reticulum.

Blood specimens from splenectomised patients contain definite Howell-Jolly bodies closely resembling reticulocytes in stage IV (Heilmeyer) with only few granules. As in smears, distinction between these cells and reticulocytes may sometimes be difficult. Occasionally the outer circumference of a red cell is stippled, but this does not confuse interpretation.

In increased bone marrow activity as in haemolytic anaemia, pernicious anaemia in remission and haemorrhagic anaemia the absolute reticulocyte count will of course be increased. A normal reticulocyte count in the presence of anaemia suggests bone marrow insufficiency. This method thus reflects the true state of erythrocyte production better than methods expressing the reticulocyte count in percentage. In addition the method is not so time-consuming as ordinary methods which require the counting of a large number of cells if the results are to be accurate.

(The skilful assistance of miss I. Erlandsson is gratefully acknowledged.)

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AN APPARATUS FOR RAPID MICRO-TITRATION OF CALCIUM IN SERUM WITH EDTA

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(Received for publication July 31, 1958.)

A description is given of an apparatus ich permits rapid and easy determination the end point in the titration of calcium h EDTA.

Principle: The end point in the titration calcium with EDTA and murexide as icator is reached the moment the color turned over to purple. By means of the cribed apparatus, this moment can be ertained and the addition of EDTA can stopped immediately. The apparatus is structed as a photometer with a light m passing a filter and the cuvette before ching the photocell. The photocell acts n a mirror galvanometer. The cuvette is nished with a magnetic stirrer keeping the ution in it well mixed.

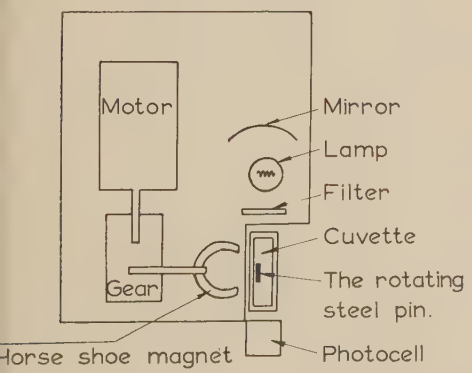

Fig. 1. Sketch of the apparatus.

Details: Source of light: A bulb, 6 Volts 0.5 peres, is connected to a 6 Volt battery. Paral- light beams are obtained by means of a con- mirror. *Filter:* Ilford 607 (Maximum trans- sion at about 600 mμ).

Cuvette: 20 mm light path, width about 5 mm (Kleincuvetten for Zeiss Pulfrich Photometer).

Photocell: A selenium photocell 3 x 6 mm is placed so it can be hit only by the light passing the lower third of the cuvette.

Stirring mechanism: A horse-shoe magnet, rotating 5 times a second, is placed just outside the one long side of the cuvette. A steel pin melted in glass, length 8 mm, diameter 2.5 mm, placed within the cuvette, is driven round by the magnet. Thereby the content of the cuvette is mixed continually.

Equipment necessary for the addition of EDTA: A micropipette is fastened vertically in such a way that its tip is placed within the cuvette. The pipette touches neither the rotating steel pin nor the walls of the cuvette. The solution of EDTA is conducted through a polyethylene tube to the pipette from a microburette. The flow rate of the EDTA solution ought to be about 0.3 ml per minute.

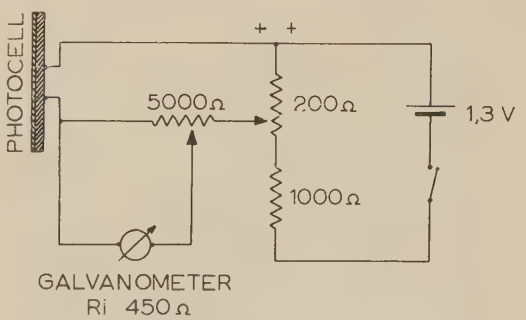

Fig. 2. The wiring diagram of the apparatus.

The electrical arrangement: Two electric circuits are connected. A constant 1.3 Volt battery is the electrical source in the right circuit, the photocell in the left. By means of the variable resistor, 200 ohms, the galvanometer is adjusted at zero. It's sensitivity is changed by the variable resistor 5000 ohms. Galvanometer: Mirror galvanometer, the

maximum deflection corresponds to about 1 microampere. The galvanometer is connected so the light spot moves to the right when the light absobtion in the cuvette increases.

Reagents:

EDTA solution: 0.186 g Ethylenediaminetetracetic acid disodium salt is dissolved in 1000 ml water.

NaOH 0.3 N: 3 g NaOH is dissolved in 250 ml water. Kept in polyethylene bottle.

Murexide solution: 10 mg murexide is dissolved in 10 ml water. The solution can be stored until about two weeks in a dark bottle in refrigerator.

Calcium standard solution: 10 mg Ca per 100 ml.

Procedure

Immediately before the analysis 1.3 ml NaOH solution, 0.1 ml (0.05 ml) serum or calcium standard solution and 0.02 ml murexide solution are placed in the cuvette together with the small glass covered steel pin. The cuvette is placed in the apparatus. By means of the variable resistor 200 ohms the light spot of the galvanometer is placed in the left part of the scale. The addition of EDTA is started. The light spot now moves to the right. At the moment this wandering halts, the end point of the titration is reached and the addition of EDTA is discontinued. By comparing the consumption of EDTA with known calcium standard solution and sera the content of calcium is estimated. It is well known that the values obtained with the EDTA method are little less than those obtained by the method of Clark & Collip (shown by Lehmann 1953, Eldjarn, Nygaard & Sveinsson 1955). The authors have determined the concentration of calcium in 70 sera both with the method of Clark & Collip and with titration with EDTA by means of the described apparatus. The concentration of calcium in the different sera ranged from 5 to 13 mg per 100 ml. It was found that an addition of 5 per cent was necessary to obtain agreement between these two methods. This is in full accordance with the conclusion of Eldjarn, Nygaard & Sveinsson (1955).

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CONSTRUCTION AND USE OF A SIMPLE FLECTOMETER FOR DETERMINATION OF HEMOGLOBIN OXYGEN SATURATION IN BLOOD

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(Received for publication February 18, 1958)

many instances it is essential to have id and simple method for determination ne hemoglobin oxygen saturation in l. As shown in the monograph of Zijl- (1951) the reflectometric methods d fulfill the most common require- s for accuracy and rapid, simple per- nce.

the following the construction and use simple and cheap reflectometer is ibed. Special attention is given to the lation, in order to reduce the analytical s due to individual factors in the blood les, especially varying concentrations of globin, and to variations in the sensi- of the apparatus.

METHODS

Description of the reflectometer.

. 1 shows a schematic diagram of the reflec- er and the light paths, and Fig. 2 shows the tometer with the top plate removed.

e light to be measured is reflected from the al side of an ordinary 10 mm Beckman glass e. The amount of light falling on the sample gulated by a movable, rectangular shutter, is adjusted by means of a calibrated micro- screw. The adjustment can be read with an acy of one hundredth of a millimeter. As the e is placed at the principal focus of a con- g lens, a constant area of the cuvette is nated, independent of the position of the r. In order to avoid heating of the photo- the lamp house is mounted on rails, separated the main body of the apparatus by an air of several millimeters.

e coupling of the photocells in a compensating minimizes variations in the readings due to ations in the lamp voltage, and the lamp can ore be run directly from the mains, without tabilization. By running the lamp at a volt- 0 per cent below its rated voltage, a longer

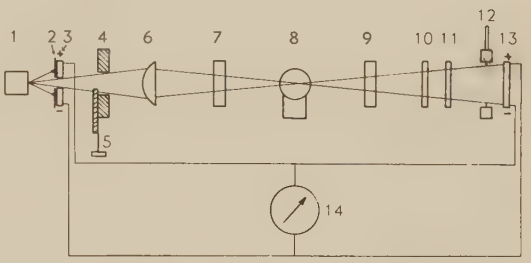

Fig. 1. Schematic diagram of the reflectometer and the light paths. 1: Cuvette with sample, 2: Filter (Ilford No. 808), 3: Measuring photocell (Megatron, Type A), with a central aperture, 4: Rectangular shutter, 5: Micrometer screw, 6: Converging lens, 7 & 9: Heat absorbing filters, 8: Light source (Osram, Type 7577, 6 V, 25 W,— *i.e.* an ordinary automobile lamp), 10: Opal glass, 11: Filter (Kodak Wratten gelatine filter No. 29), 12: Iris diaphragm, 13: Compensating photocell (Megatron, Type A), 14: Galvanometer (Multiflex, 300 ohm, sensitivity $8 \cdot 10^{-9}$ amp./mm).

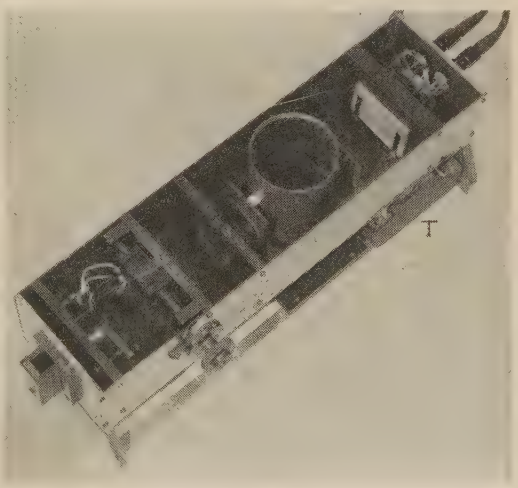

Fig. 2. Reflectometer with top plate removed. (T: Transformer 230/6 V.)

period of approximately stable light output and a slower blackening of the lamp envelope is obtained.

The reflectometer measures 13 x 13 x 40 cm.

Standardization.

The reflectometer was standardized by means of two reflection standards, a red enamel plate, with reflection reading slightly higher than for fully saturated blood, and a brown ebonite plate, with reading somewhat lower than for completely desaturated blood. The reflection values of the standards were determined against the reflection from the sample cuvette, filled with a non-reflecting solution:

The cuvette, filled with India ink, is placed in the reflectometer, the calibrated shutter is opened fully, and the galvanometer is set at zero by means of the iris diaphragm. The cuvette is replaced by the enamel standard, the galvanometer is zeroed by adjusting the calibrated shutter, and the position of the shutter is read (R_1). Then the ebonite plate is inserted and the shutter position is determined in the same way (R_2). After repeated determinations the mean values, $\bar{R}_1$ and $\bar{R}_2$, are calculated.

The reflectometer was adjusted and controlled before each analysis:

The red reflection standard is inserted, the calibrated shutter is set to $\bar{R}_1$, and the galvanometer is zeroed by means of the iris diaphragm. The ebonite standard is inserted, and its actual R_2 value is read. Deviations of R_2 from $\bar{R}_2$ indicate variations in the sensitivity of the reflectometer which, if significant, can be corrected for.

Material.

All investigations were performed in heparinized blood from nonsmokers in the postabsorbtive phase. The blood to be analyzed for hemoglobin oxygen saturation was kept in anaerobically sealed Luer syringes. Samples were taken from these syringes and equilibrated to 20° C immediately before the analyses were begun.

Analytical procedure.

1.5 — 2.0 ml of blood is injected through a short piece of rubber tubing into a 5 ml Luer syringe, containing an equal amount of the stabilizing solution recommended by Zijlstra (1951)*, and 0.1 ml of mercury.

After thorough mixing for 1 minute, the syringe is fitted with a straight cut-off cannula, the tip of the cannula is placed on the bottom of the cuvette, and the cuvette is filled with the diluted blood. The reflectometer is adjusted, and the reflection is read 1 minute after the filling of the cuvette.

* 2 g Na-chloride, 0.3 g Na-salicylate and 0.05 g Na-cyanide per 100 ml.

After rinsing of the cuvette with stabilizing solution, another sample can be introduced at once without drying of the cuvette.

By gentle filling of the cuvette only the upper layer of the blood gets into air contact, and employing the above volumes, the readings remain constant for more than 5 minutes.

No rapid changes in the readings due to sedimentation have been observed, even in blood samples with a sedimentation rate of 100 mm.

Determination of reflection from completely saturated and desaturated blood.

The reflection from blood samples saturated with oxygen was determined as described above. Complete saturation was obtained by rotating 6 — 8 ml of blood for 20 minutes in a 300 ml tonometer flushed with air at intervals.

The reflection from completely desaturated blood was determined either *directly*, after mixing equal amounts of Ziljstra's desaturation solution* and blood in a Luer syringe for 3 minutes, or *indirectly* by extrapolation, after simultaneously measuring reflection and determining oxygen saturation by spectrophotometry (Klungsøyr & Støa, 1954; Refsum, 1957) in a series of blood samples with high and low degrees of oxygen saturation. This was done in 16 samples from 6 donors with an average hemoglobin concentration of 13.9 g/100 ml.

Comments on the determination of reflection from desaturated blood.

The reflection values found after desaturation of the blood with sodium dithionite according to Ziljstra, decreased rapidly for 5 — 10 minutes after the filling of the cuvette, and even the initial, highest values were clearly *lower* than those found by extrapolation. When inserting the mean values obtained with sodium dithionite, respectively by extrapolation in the formula for calculation of hemoglobin oxygen saturation, the differences between the results amounted on an average to $0.075 \cdot (100—s)$, s being the saturation calculated with the extrapolated value. It appears that the differences are of less importance at high degrees of saturation. The discrepancies between the result

* Stock solution: 2 g Na-tetraborate and 0.2 g Na-salicylate per 100 ml. The final desaturation solution was prepared within one hour before use by mixing 20 ml of the stock solution with 0.2 g Na-dithionite (Merck) in a Luer syringe, anaerobically sealed.

nly partly be explained by the mixing e blood to be analyzed with a stabilizing ion containing physically dissolved oxy- and to the air contact during the filling e cuvette.

adding more sodium dithionite to the uration solution, thus increasing the ity of the solution, *higher*, but still un- e reflection values were found.

should be mentioned that these obser- ns are in contrast with observations e in a previous study, using solid sodium onite as reducing agent in determination emoglobin oxygen saturation in *hemo-* whole blood by *spectrophotometry* sum, 1957): The readings remained ant for at least half an hour, and were ically the same whether 6 or 18 mg m dithionite was used for reduction per of blood. Using constants determined reduction with sodium dithionite in calculation formula, good agreement was d between the spectrophotometric and ometric values.

ulation of the hemoglobin oxygen ration.

s there is a rectilinear relationship be- n the logarithm of the amount of light cted from a blood sample and the hemo- in oxygen saturation (Kramer, 1935), percentage oxygen saturation can be cal- ted from the formula (1):

$$\text{centage } HbO_2 = 100 \cdot \frac{\log R_x - \log R_0}{\log R_{100} - \log R_0}$$

being the reading of the actual analysis, and R_0 the readings of blood with 100 0 per cent saturation respectively. Ac- ing to Ziljstra (1951) R_{100} is fairly con- when blood samples from various rs are investigated, provided that the oglobin concentration of the diluted d is higher than 6 g/100 ml, while R_0 es slightly more. Consequently it should ossible to determine R_{100} and R_0 once for all, and thus simplify the analytical edure. In our experiments, however, ng equal amounts of blood and stabi- lizing solution, both R_{100} and R_0 were found to vary considerably from blood to blood, especially due to different hemoglobin concentrations, and over the whole physiological range of hemoglobin concentrations. When inserting the mean values of $\log R_{100}$ and $\log R_0$ for blood samples with an average hemoglobin concentration of 13.6 g/100 ml in the formula above (1.3349 and 1.2183) and substituting $\log R_x$ with the mean $\log R_{100}$ values of blood samples with average hemoglobin concentrations of 8.9 and 17.9 g/100 ml respectively (1.3301 and 1.3372), the calculated hemoglobin oxygen saturations were 96 and 102 per cent respectively. The same magnitude of error was found when $\log R_x$ values of samples with other degrees of oxygen saturation were inserted.

But while both R_x, R_{100} and R_0 differed from blood to blood, the *differences* $\log R_{100} - \log R_0$ were found to be essentially constant, and the following formula was adopted (2):

$$\text{Percentage } HbO_2 = 100 \cdot \frac{\log R_x - [\log R_{100x} - (\log \bar{R}_{100} - \log \bar{R}_0)]}{\log R_{100} - \log R_0}$$

$\log R_{100} - \log R_0$ being a constant, determined for the reflectometer (cf. Ziljstra, 1951), R_x the reading of the actual sample, R_{100x} the reading of the actual sample after saturation with oxygen, and the contents of the square brackets consequently the actual $\log R_0$ value.

The formula implies that the calibration curves on semilog paper for the different blood samples have a constant slope, and thus routine determinations of R_0 are avoided. In addition to the reduction of errors due to individual factors in the blood samples, the formula has the advantage that errors due to variations in the sensivity of the reflectometer are practically eliminated at high levels of oxygen saturation and considerably reduced at low levels. Observations concerning the relationship between hemoglobin concentration and reflection values have also been published by Kramer, Graf & Overbeck (1956).

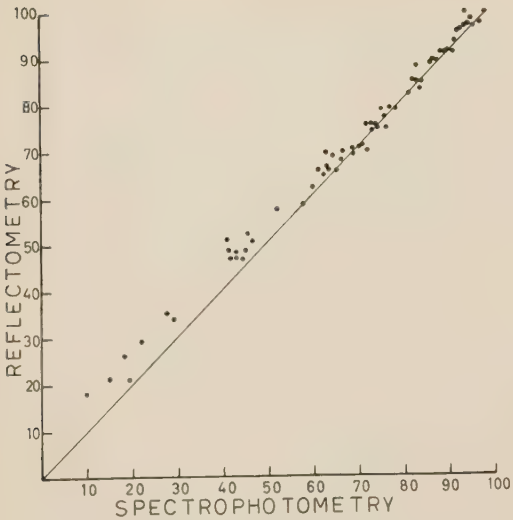

Fig. 3 a. Relation between the reflectometrically and spectrophotometrically determined values of hemoglobin oxygen saturation in per cent, when formula 1 is used for the calculation, the mean $\log R_0$ value determined by means of sodium dithionite inserted.

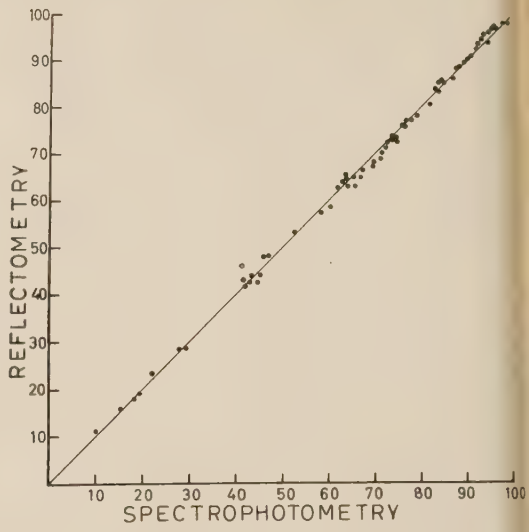

Fig. 3 b. Relation between the reflectometrically and spectrophotometrically determined values of hemoglobin oxygen saturation in per cent, when formula 2 is used for the calculation, the $\log R_0$ value determined by extrapolation inserted.

RELIABILITY OF THE METHOD

To test the reliability of the method the hemoglobin oxygen saturation was determined in 66 blood samples from 23 different donors, both by reflectometry and spectrophotometry (Klungsøyr & Støa, 1954; Refsum, 1957). The hemoglobin concentrations of the samples varied between 8.7 and 19.3 g/100 ml. Fig. 3 a shows the relation between the reflectometric and spectrophotometric results found, applying formula 1 for the calculation, the mean values of $\log R_{100}$ and $\log R_0$, determined after desaturation with sodium dithionite, inserted. Fig. 3 b shows the relation found with formula 2, the same $\log R_{100}$ value, the $\log R_0$ value determined by extrapolation, and the actual $\log R_{100x}$ value inserted. Table I shows the differences between the reflectometric and spectrophotometric results demonstrated in Fig. 3 b.

During the investigation, the range of the variations in the sensitivity of the reflectometer, as expressed by the deviations of the actual R_2 from R_2 (cf. standardization), corresponded to a maximum possible error in the hemoglobin oxygen saturation of ± 1.7 per cent at a saturation level of 50 per cent,

Table I. *Differences between the reflectometrically and spectrophotometrically obtained values of hemoglobin oxygen saturation in per cent in 65 samples, when formula 2 is used for the calculation, the $\log R_0$ value determined by extrapolation inserted. (See Fig. 3 b.)**

Hemoglobin oxygen saturation	Number of determinations	Mean	SD	Range
0-100	65	+0.1	1.4	—2.5 — +2.5
80-100	23	+0.6	1.0	—1.6 — +2.2
60- 80	25	—0.7	1.2	—2.5 — +2.0
0- 60	17	+0.3	1.3	—2.1 — +2.5

* One of the values in Fig. 3 b, marked by an open circle, with difference between the results of 5.2 per cent, is not included in the table.

± 3.4 per cent at a level of 0 per cent. corrections were made.

o test the reproducibility of the results, hemoglobin oxygen saturation was deter- ed successively in 10 samples of blood n the same pool. The mean value, range standard deviation in one series were ectively 83.2, 82.7-83.7 and 0.3 per cent, nother 38.9, 38.0-39.9 and 0.6 per cent. larger range and standard deviation at lower saturation are due to the increased uence of variations in the sensitivity of reflectometer and of air contact during filling of the cuvette. The variations in sensitivity of the reflectometer, however, e far less during this investigation than ribed in the previous paragraph.

SUMMARY

he construction and use of a simple re- ometer for the determination of hemoglo- oxygen saturation in blood is described. using two barrier layer cells in a compen- on circuit and a calibrated shutter in one he light paths for adjustment, variations he instrument readings due to fluctuations he lamp voltage and uneven illumination the photocells are minimized, and the p of the reflectometer can be run direct- rom the mains without any stabilization. A calculation procedure, reducing analytical errors due to individual factors in the blood samples, especially different hemoglobin concentrations, and variations in the sensitivity of the reflectometer is demonstrated.

The determination of the zero-saturation values by means of sodium dithionite is briefly commented upon. In a series of determinations of hemoglobin oxygen saturation the values found by the presented reflectometric method compared favorably with those found by a spectrophotometric method.

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QUANTITATIVE ESTIMATION OF MANNOSE, FUCOSE, AND RIBOSE ON FILTER PAPER

Using paper chromatography it is possible to separate mixtures of carbohydrates. A quantitative method for determination of the carbohydrate content in isolated spots of lactose, galactose, glucose, arabinose, and xylose was described by Date (1958). In the present paper it is demonstrated that the same procedure may be used for the quantitative estimation of mannose, fucose, and ribose, which are all present in biological fluids (urine and blood plasma).

In the same way as described by Date (1958) spots containing between 15—800 μg D-mannose (Pfanstiehl), L-fucose (Hoffman-La Roche), and D-ribose (Pfanstiehl) were placed on Whatman No 1 filter paper, sprayed with aniline citric acid reagent, heated, eluated, and read on the spectrophotometer.

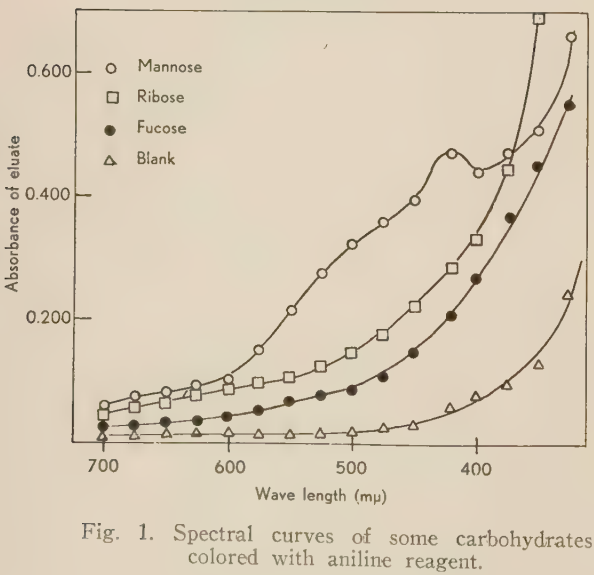

Fig. 1. Spectral curves of some carbohydrates colored with aniline reagent.

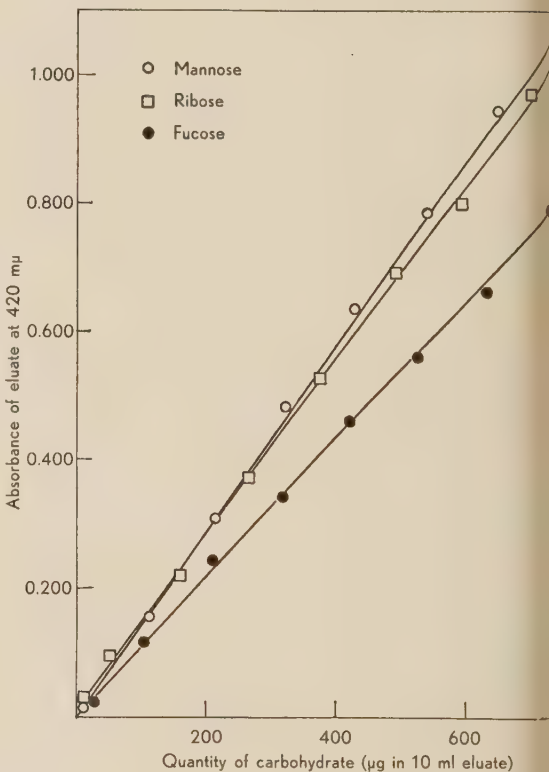

Fig. 2. Standard curves of the three carbohydrates.

The *spectral curves of the carbohydrates* are given in Fig. 1. Only mannose gives a peak at 420 mμ, but for practical reasons it was chosen to read all three carbohydrates at 420 mμ.

The *relationship between amount of carbohydrate and the absorbance* in the range 15—800 μg is given in Fig. 2. An approximation to proportionality was found for ribose in the range 100—500 μg, and for mannose and fucose from 15—800 μg.

Table I. *Recovery experiments.*

...o-ate	Quantity added (μg)	Recovery in per cent	S. D. in per cent
	100	104	6
...ose	300	97	4
	500	94	5
	100	102	4
...cose	300	100	2
	500	96	2
	100	99	4
...nnose	300	101	3
	500	102	3

...he *color yield of the carbohydrates on ...basis relative to galactose* (I_c) *was deter-*...ed by simultaneous analysis of spots con-taining 100—500 μg mannose, fucose or ribose and 125 μg galactose. I_c mannose = 0.67, I_c fucose = 0.55, and I_c ribose = 0.70.

The results of *recovery experiments* on spots containing different amounts of carbohydrate are summarized in Table I.

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...partment of Biochemistry,
...iversity of Århus, Denmark.

...gust 11, 1958.

SUMMARY

The previously published method for estimation of lactose, galactose, glucose, arabinose, and xylose has been extended to include mannose, fucose, and ribose. The color yield from the carbohydrates relative to galactose (I_c) was determined. I_c mannose = 0.67, I_c fucose = 0.55, and I_c ribose = 0.70.

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J. W. Date.

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SERUM BILIRUBIN AND THE YELLOW COLOR OF ICTERIC SERUM

An investigation recently published by the author (Fog 1958) demonstrates a discrepancy between the serum color and the amount of bilirubin in icteric sera. This problem has since been reinvestigated with an improved technic.

Serum color and serum bilirubin have been determined (as described by Fog 1958) in 24 icteric sera from cases of hepatic or posthepatic jaundice (group I), in 13 icteric sera of the prehepatic type (group II) and in 29 normal sera wherein bilirubin was dissolved (Fog 1958) with the corresponding serum without bilirubin as a blank (group III). Serum bilirubin was calculated as per cent of the amount of bilirubin corresponding to the serum color.

Table I.

Groups	Number of samples	Bilirubin as per cent of calculated bilirubin Mean
I Hepatic or posthepatic icterus	24	81.1 ± 1.1*)
II Prehepatic icterus	13	96.3 ± 1.9
III Bilirubin dissolved in normal serum	29	100.6 ± 1.0

*) Standard error of mean.

The means compared by the "t" test show an extremely small probability ($P \ll 0.001$) of group I and III belonging to the same population while group II and III are only slightly different ($P = 0.05$).

From Table I it is seen that sera of the prehepatic type (group II) have a mean only slightly different from that obtained with bilirubin dissolved in normal serum (group III). In hepatic and posthepatic sera (group I) are serum bilirubin responsible for about 80 per cent of the serum color only. This result leads the author to suppose that the discrepancy between serum color and serum bilirubin may be explained as a result of the conjugated or acid bilirubin being the more dominant.

Billing, Cole and Lathe and others have shown in numerous publications (ref. Billing & Lathe 1958) that sera classified in group I mainly contain conjugated bilirubin while bilirubin as an acid is the principal pigment in group II and in normal sera. Therefore, the suggestion set forth by the author may be proved if conjugated bilirubin dissolved in human serum is more intensely colored (has a greater molar extinction coefficient) than acid bilirubin in serum. Experiments

aling with this problem have yet not been iished.

Sera from patients without jaundice must ve a relatively great background yellow or. The serum colors of these sera are due inly to bilirubin and fluctuations to bili- bin only (Fog 1958). This favours the lief that the discrepancy is due to a mix- re of two or more "bilirubin pigments".

Icteric sera have been diluted to fixed serum ors (4.5 — 8 — 12 in the Meulengracht scale) ving triple analyses. The correlation between $E^{corr.}_{406}$ and E^{azo}_{600} (indicating serum color and serum bilirubin respectively) leads to a slightly curved correlation line. Therefore a second degree equation fitted by least squares has been used to calculate the amount of serum bilirubin as per cent of serum color when bilirubin (Hoffmann La Roche) dissolved in samples of normal serum is used as one hundred per cent.

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iiversity Clinic, Medical Department B, kshospitalet, Oslo, Norway.

J. Fog.

ptember 1958.

INHIBITION OF RADIOVITAMIN B_{12} ABSORPTION BY ETHYLENEDIAMINETETRAACETATE (EDTA) AND ITS REVERSAL BY CALCIUM IONS

Intrinsic factor preparations have been found to stimulate the *in vitro* uptake of vitamin B_{12} by liver slices. Calcium ions are necessary for this process, which is inhibited by EDTA (Herbert, 1958). To study whether calcium ions are needed for intestinal B_{12} absorption in man, we administered EDTA to test subjects together with vitamin $B_{12}Co^{60}$, the absorption of which was subsequently determined by the urinary radioactivity test (Gräsbeck, 1956). The oral dose was 1 $\mu g = 0.2$ μc $B_{12}Co^{60}$, and the urine was collected for 24 hours.

The test was performed twice, equal amounts (2—10 g) of sodium-EDTA being mixed with the radiovitamin on both occasions. On the second occasion calcium lactate tablets were administered simultaneously. The amount of calcium given exceeded the binding capacity of the EDTA dose. In many cases an ordinary urinary radioactivity test without EDTA was performed in addition.

The results are presented in Table I. It is seen that EDTA alone inhibited radiovitamin B_{12} absorption, whereas simultaneous administration of calcium abolished the inhibition, the values being about the same as when no EDTA was given.

Vitamin B_{12} absorption is often impaired in steatorrhea, and administration of intrinsic factor does not normalize it (Callender & Evans, 1955). The situation might be exactly analogous to the EDTA effect: The fatty acids bind calcium in the form of insoluble calcium soaps. This mechanism is thought to be responsible for the impaired calcium absorption in steatorrhea (Korelitz & Janowitz, 1957). That this hypothesis may be correct was indicated by the finding that administration of calcium lactate increased the Schilling-test value in the two cases of steatorrhea so far studied (rise from 0.3 and 5.5 to 19.4 and 8.7 per cent, respectively).

Table I. *Effect of EDTA on the Schilling-test.*

Case No.	Urinary radioactivity (per cent of oral dose)		
	EDTA	EDTA + calcium	No EDTA
1.	1.0	10.1	-
2.	1.8	8.2	-
3.	1.4	5.1	-
4.	3.1	10.8	-
5.	3.2	14.2	10.5
6.	2.3	10.3	11.9
7.	3.8	14.4	9.9
8.	2.3	8.3	10.9
9.	2.7	11.3	10.2
10.	5.1	23.0	26.8
11.	16.1	22.4	29.1
12.	10.6	17.1	17.8

October 23, 1958.

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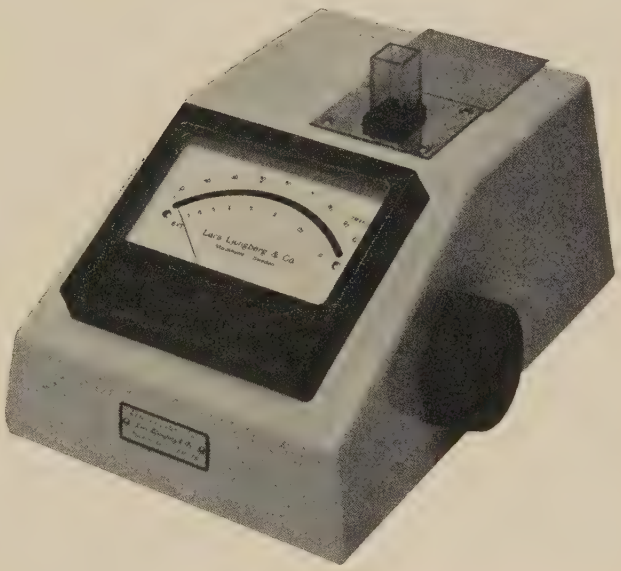

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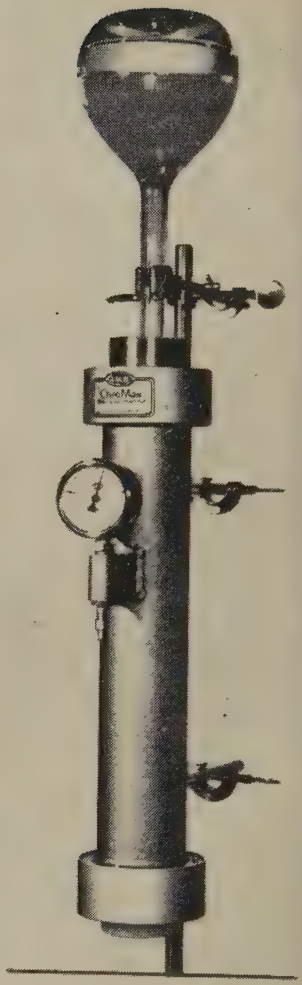

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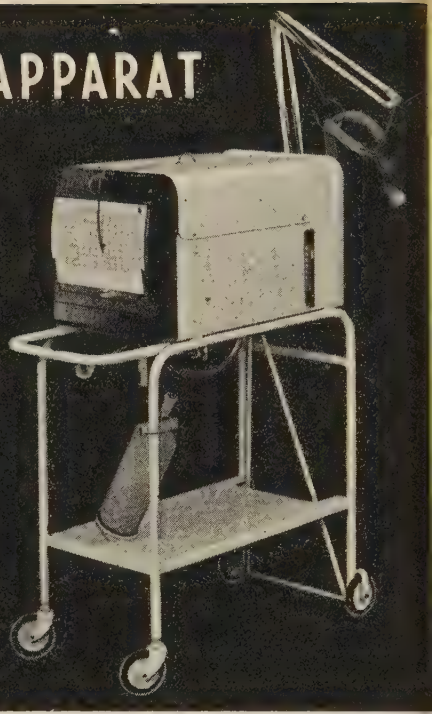

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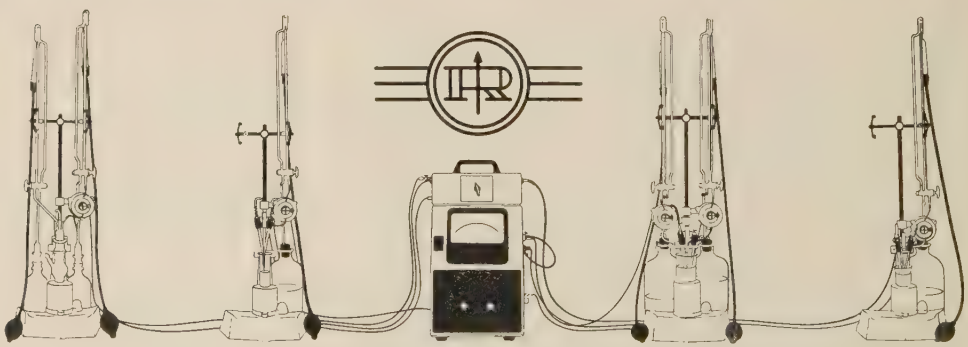

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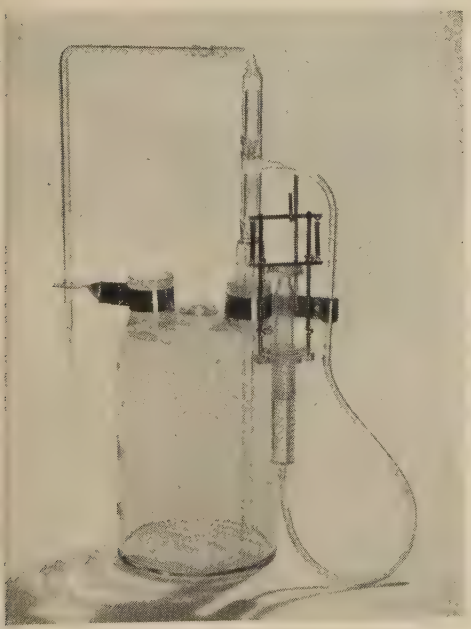

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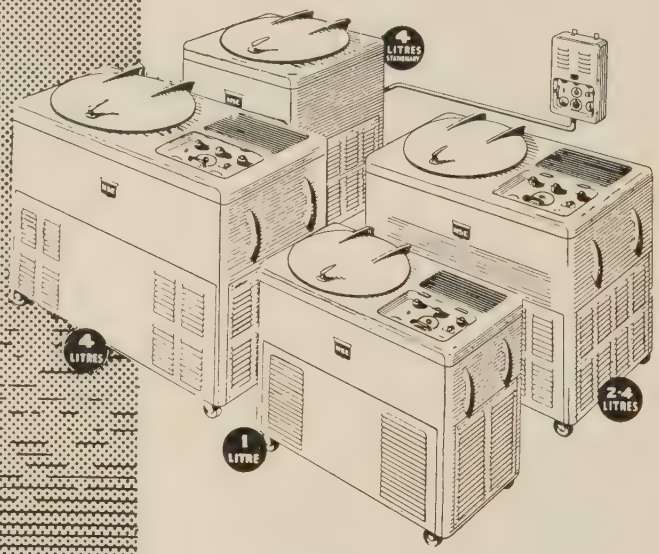

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PRINTING ARRANGEMENTS BY OSLO UNIVERSITY PRESS, OSLO, NORWAY

PRINTED BY A. W. BRØGGERS BOKTRYKKERI A/S, OSLO